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Full Length Research Paper

Agrobacterium mediated genetic transformation and regeneration in elite rice (*Oryza sativa* L.) cultivar BRRI dhan56

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***Agrobacterium*-mediated genetic transformation of rice (*Oryza sativa* L.) cultivar BRRI dhan56 was carried out in this study. *Agrobacterium tumefaciens* strain LBA 4404, which harbors the plasmid pIG121 that carries the genes for ß-glucuronidase gene, served as a reporter gene in the histochemical assay and the neomycin phosphotransferase II (*NPT II*) gene for the identification of resistance to kanamycin was used for genetic transformation. Twenty days old embryogenic calli from mature embryos of highly regenerating rice cultivar BRRI dhan56 were used to co-cultivate with 0.8 to 0.9 OD_{600} *Agrobacterium* for 25 min and the cultured was continued on agar medium for this study. The transformed colonies were selected by using 50 mg/L kanamycin and 50 mg/L rifampicin and confirmed by colony PCR. The PCR positive colonies were isolated to transform by using calli of indica rice cultivar BRRI dhan56. Putative leaf and root segments from plantlets obtained from transformation experiment with the plasmid pIG121 were GUS positive. Integration of the introduced gene into the genome was demonstrated by PCR. The maximum transformation efficiency of 32% was obtained by using 500 mg/L cefotaxime as a bacteriostatic agent to inhibit growth of *Agrobacterium*. In this study, 100 µM acetosyringone in co-cultivation medium and co-cultivation for 3 days were the optimum conditions for maximum transformation. The expression of GUS gene revealed that the calli were successfully transformed. The results of this study would be an effective tool for crop improvement and gene-function studies on the model monocot plant rice.**

Key words: *Agrobacterium, Oryza sativa* L., acetosyringone, β-glucuronidase, cefotaxime, plasmid, phosphotransferase, rice, transformation.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most significant cereal crops of Poaceae family cultivated for more than 10,000 years (Sasaki, 2005). It was the first major cereal crop regenerated into whole plant from tissue culture (Vasil, 1983). It is an economically very important crop in the world, with more than half of the world's population depending on it as a primary staple food (Lu, 1999). Its cultivation is concentrated for the most part in Asian

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countries, which together make up approximately 90% of the rice cultivation area in the world. Numbers of rice consumers are increasing at the rate of 1.8% every year (Islam et al., 2015). It is estimated that rice production has to be increased 50% by 2025 (Khush and Virk, 2000). Approximately 80% of the world rice production is based on indica rice varieties which are cultivated under subtropical and tropical conditions as long grain rice and thus securing a unique position in agriculture (Khush, 1997; Abbasi et al., 2010).

In recent years, some important agronomic traits of rice have been improved through biotechnological approaches (Hao et al., 2009; Skamnioti and Gurr, 2009; Cheng et al., 1998). Genetic transformation has become a valuable tool in targeted improvement and gene function studies in rice (Xu et al., 2012). Most indica rice varieties were adapted to by *Agrobactrerium*-mediated genetic transformation (Chan et al., 1992; Rashid et al., 1996; Khanna and Raina, 2002; Supertana et al., 2005; Ignacimuthu and Arockiasamy, 2006) involve regeneration of plants from transformed embryogenic calli, anther calli (Jiang et al., 2004; Arnold et al., 1995), and protoplasts. *Agrobacterium*-mediated transformation has significant advances of such as introduction of fewer copies of genes into the plant genome, high co-expression of introduced genes, transformation of relatively large segments of DNA and high fertility of transgenic plants (Cheng et al., 1997; Hiei et al., 1994; Datta et al., 1992). *Agrobacterium tumefaciens* mediated transformation has been well established, owing to its simplicity, low cost, as well as low copy number of transgene integration. However, the transformation efficiency is very low in the indica rice varieties. Most indica rice varieties also show a low rate of callus growth and low regeneration frequency in conventional culture (Nishimura et al., 2007; Larkin and Scrowcroft, 1981). Now *Agrobacterium*-mediated rice transformation has been successful in many cultivation of japonica, indica and javanica (Aldemita and Hodges, 1996; Hiei et al., 1994; Dong et al., 1996).

There are several factors which are involved in *Agrobacterium*-mediated gene transfer into rice. During co-cultivation, the addition of acetosyringone, media with 2,4-D, acidic pH and high osmotic pressure have been reported to be significant for the induction of *vir* gene expression on Ti plasmid (Turk et al., 1991; Usami et al., 1988). Most reports suggested to use actively growing, embryogenic calli derived from rice mature seed or immature embryos as receptors to be infected by *A. tumefaciens* (Hiei et al., 1997). In addition, shoot apices of tropical japonica have also been used as gene receiver (Park et al., 1996; Rogers and Bendich, 1994). In the present study, we report establishment of an efficient plant regeneration system from mature seeds of indica rice applicable to BRRI dhan56 as explants using different growth regulators and demonstrate their amenability to *Agrobacterium*-mediated transformation.

MATERIALS AND METHODS

Plant materials

Indica rice (*O. sativa* L.) cultivar namely BRRI dhan56 was used as explants for study of *in vitro* regeneration and genetic transformation. Seeds were collected from Bangladesh Rice Research Institute (BRRI) Regional office, Rajshahi, Bangladesh.

Preparation of explants

Mature seeds of indica rice cultivars namely BRRI dhan56 were dehusked carefully and separately. Dehusked seeds were taken in beakers having distilled water and 1 or 2 drops of Twin-80 were added in the distilled water. The mixture was shacked for 5 min with orbital shaker. Seeds were then washed with distilled water for several times to remove the effect of Twin-80. Then seeds were bought in front of laminar air flow and surface sterilized with 0.1% (w/v) $HgCl_2$ solution for 5 min by vigorous shaking twice. Treated seeds were rinsed with distilled water several times to remove mercuric chloride completely and blot dried on to a filter paper.

Callus induction

For callus induction, three seeds were inoculated per test tube on callus induction medium (MCI) and incubated at 26 ± 2°C in dark. MCI was prepared using by basal MS salts containing all vitamins (Murashige and Skoog, 1962) supplemented with 2.5 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D), 0.50 mg/l naphthalene acetic acid (NAA), then the medium was solidified 0.8% agar before autoclaving 21 min at 121°C and 1.07 kg cm^{-2} and pH of the medium was adjusted to 5.8 before addition of agar. After 20 days, non-embryogenic calli (compact, non-friable calli that develop root like structures) were discarded and only embryogenic calli were selected. These embryogenic calli were cut into around 3 equal halves, then sub-cultured again onto fresh MCI and kept for 4 days (dark, 26 ± 2°C) before transformation with *A. tumefaciens*.

Agrobacterium strain

The bacterial (*A. tumifaciens)* strain LBA4404 with the binary plasmid pBI121 strain was used for infection in the transformation. The binary vector pBI121 has the background of pBIN19. It contains a reporter gene GUS (β-glucurunidase) driven by a CaMV35S promoter and NOS terminator and a selectable marker gene *nptII* fused between promoter and terminator encoding for the encoding for the enzyme neomycin phosphotransferase conferring kanamycin resistance.

Culture of *Agrobacterium* strain

Primary culture of *Agrobacterium* strain was prepared by inoculating single colony from a freshly streaked plate which contains 5 ml of autoclaved liquid YEP medium (10 g/l bactopeptone, 10 g/l yeast extract, 5 g/l sodium chloride and pH 7.0) supplemented with 25 mg/l streptomycin, 50 mg/l rifampicin and 50 mg/l kanamycin. The culture was incubated for 20 hours on a rotatory incubator shaker at 120 rpm in dark at 28°C. Secondary culture of *Agrobacterium* strain was prepared in a 500 ml baffled flask containing 50 ml YEP medium (supplemented with same antibiotics as used for primary culture) by adding together 0.4% of the primary culture and grown under same environment. Once the OD_{600} reached ~1.0, *Agrobacterium* cells were pelleted by centrifugation at 8000 × *g* for 15 min at 4°C. The cells were re-suspended in MS re-suspension medium containing 150 µM acetosyringone (MS salts, 6 8 g/l

sucrose, 36 g/l glucose, 3 g/l KCl, 4 g/l $MgCl_2$ and pH 5.2) to adjust the OD_{600} of the bacterial suspension to 0.3.

Co-cultivation and selection of transformed calli

Sub-cultured (four days) embryogenic calli were collected and Agro-infected by immersing them in the *Agrobacterium* culture (LBA4404) for 20 to 25 min with blinking gentle shaking at 50 rpm. The Agro-infected calli were dried on sterile filter paper for 5 to 6 min. Calli were then transferred to the co-cultivation medium containing 10 g/l glucose, pH 5.2, 150 µM acetosyringone (Ali et al., 2007) and incubated at 26 ± 2°C in the dark for around 48 h. Once slight growth of *Agrobacterium* appeared around most of the calli, the calli were rinsed 8 to 10 times with 500 mg/l cefotaxime in sterile distilled water, dried on sterile filter paper and transferred onto first selection medium (MCI containing 500 mg/l cefotaxime and 100 mg/l kanamycin) and incubated for 13 days at 26 ± 2°C in dark. After the first selection, brown or black calli were removed and only creamish healthy calli were shifted to the fresh MS media for second selection and maintained at 26 ± 2°C in dark. After second selection for 11 days, micro calli could be observed which were finally transferred to fresh MS media for third selection and allowed to proliferate for five days at 26 ± 2°C in dark.

Regeneration of transformed calli

After third selection, black or brown microcalli were discarded and only granular 'macrocalli' were transferred onto MS media containing two or three growth regulators viz. 6 benzylaminopurine (BAP), kinetin, naphthalene acetic acid (NAA), pH 5.8; 250 mg/l cefotaxime and 50 mg/l kanamycin added after autoclaving. These microcalli were incubated at 26 ± 2°C in dark for seven days for the first phase of regeneration. During the second phase of regeneration, these were shifted to fresh same regeneration medium and incubated in light for four days. The regeneration frequency was calculated as per the formula: Regeneration frequency (%) = [(Number of microcalli regenerating shoots) / (Number of microcalli incubated)] x 100.

For development of vigorous roots, the regenerated shoots were shifted to rooting media and finally transfer to soil pot for hardening.

Histo-chemical GUS assay

Infected calli after co-cultivation as well as regenerated shoots of putative transformants were assayed for transient GUS activity according to the procedure described by a researcher (Jefferson, 1987). Tissue segments were immersed overnight in assay buffer containing 20% methanol, 500 µM potassium ferrocyanide, 500 µM potassium ferricyanide, 5 mM EDTA, 50 mM sodium phosphate buffer (pH 7), 0.5% triton X- 100 and 20 mM X-Glu. Then treated explants were transferred to 95% ethanol for preservation and the explants were observed for visualization of color products under zoom stereomicroscope.

DNA isolation and PCR analysis

Genomic DNA was collected from transformed and untransformed calli by using the CTAB method. PCR analyses were carried out by using two GUS primers namely; forward 5' CCTGTAGAAACCCCAACCCG 3' and reverse 5' TGGCTGTGACGCACAGTTCA 3' for amplification of GUS gene transformants. The reaction mixture (20 µl) of PCR consists of 1.0 µl DNA template, 2.0 µl 10× buffer, 1.0 µl (2.5 mM) dNTPs, 2.0 µl (25 mM) $MgC1_2$, 1.0 µl of each primer (F/R), 0.4 µl *Taq* DNA polymerase and ddH_2O 13 µl. Reaction procedures were carried out at 94°C for 4 min and followed by 25 cycles at 94°C for 1 min, 56°C for 45 s and 72°C for 1 min. After the final cycle, the reactions were maintained at 72°C for 5 min before completion. Finally, PCR products were analyzed on 1% agarose gel with 0.5× TBE buffer.

RESULTS AND DISCUSSION

Callus induction

Transformation of rice using *Agrobacterium* mediated methods and subsequent regeneration are dependent on several factors such as the choice of explants, hormonal concentrations of the medium used and nutritional supplements highly affects the callusing and regeneration(Lin et al., 1995; Katiyar et al., 1999). A reporter (Hiei et al., 1994) reported that scutellum derived callus was the most amenable explants for *Agrobacterium* mediated transformation. In the present investigation, seeds of elite indica rice cultivar BRRI dhan56 was used as explants for *Agrobacterium* mediated transformation. Calli were produced by using different types of hormonal concentrations of 2, 4-D (1.5 to 3.0 mg/l), NAA (0.5 to 2.0 mg/l), 2, 4-D + NAA (1.5+1.0 to 3.0+1.0 mg/l), 2, 4-D + BAP (1.50+0.10 to 2.50+.50 mg/l) and NAA + BAP (1.0+0.20 to 2.0+1.50 mg/l) were used and 2, 4-D + NAA (2.0+1.0 mg/l) was showed the highest result where 95.22% explants produced calli in BRRI dhan56 (Figure 1A and B). All the recorded data are given in Table 1. With the increase of concentration of 2, 4-D above 2.0 mg/L the callus induction efficiency was reduce in rice cultivar. This indicate that the use of low concentration of 2, 4-D was enough for production of high amount of callus in rice. It was revealed from the results of tissue culture experiments that BRRI dhan56, showing the best callus induction with combinations of 2, 4-D and NAA.

Similar result for the callus induction in rice were also reported by others (Rashid et al., 2003; Islam et al., 2013; Roly et al., 2013, 2014; Islam et al., 2014a; Islam et al., 2014b). After 12 days, calli of BRRI dhan29 was sub cultured in MS medium containing different concentrations of 2, 4-D for development of Embryogenic calli (Table 2). The highest Embryogenic calli (95.22%) showed MS media with 2, 4-D + NAA (2.0+1.0 mg/l). Different days (10, 15, 20, 25 and 30 days) of calli were tested as suitable for transgenic rice BRRI dhan56. For *Agrobacterium*-mediated genetic transformation, twenty days of old calli are suitable for transgenic rice BRRI dhan29 (Figure 1C and D). Regeneration responses of embryogenic calli derived from mature seeds were influenced with the concen-trations and combinations of 2,4-D and NAA present in the regeneration media. Similar results were reported by some researcher (Pandey et al., 1994; Islam et al., 2013) who observed a high regeneration frequency for the medium containing high levels of 2, 4-D and NAA and after a certain levels 2, 4-D and NAA, callus induction frequency decreased.

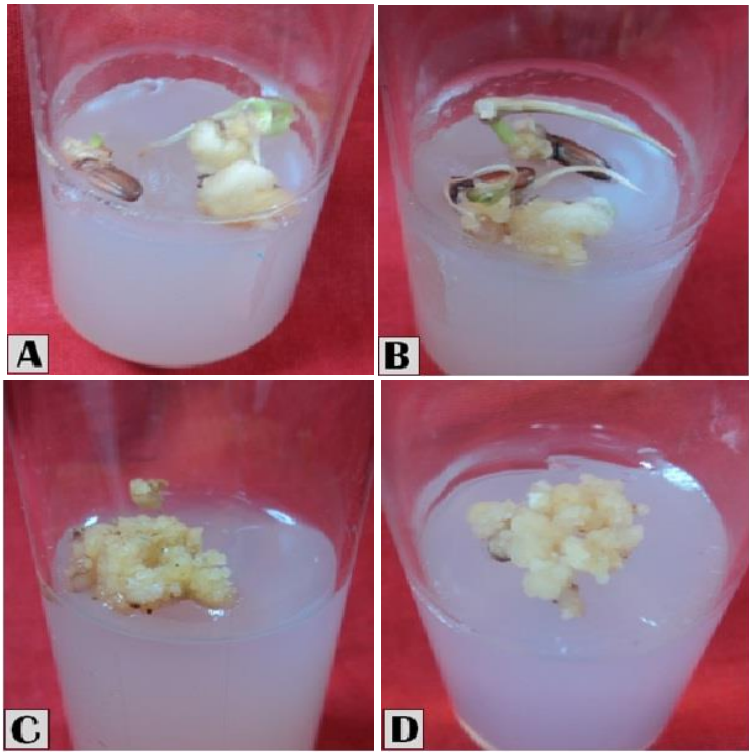

Figure 1. Different types of calli from mature embryo in elite rice cultivar BRRI dhan29: **(A)** and **(B)** Ten days old callus, **(C)** and **(D)** Embryogenic calli derived from mature embryo on callus induction medium.

Table 1. Effects of different hormonal combinations (2, 4-D, NAA, 2, 4-D + NAA, 2, 4-D + BAP and NAA + BAP) for callus formation from zygotic embryo of BRRI dhan56.

PGRs	PGR concentrations (mg/l)	Percentage explants induced calli	Color of calli	Morphological features of calli
2,4-D	1.5	90.66±0.21	Pale yellow	Compact
	2.0	93.44±0.32	Pale yellow	Compact
	2.5	90.66±0.50	Pale yellow	Compact
	3.0	87.59±0.41	Pale yellow	Compact
NAA	0.50	41.75±0.31	Pale yellow	Compact
	1.00	62.86±0.09	Pale yellow	Compact
	1.50	71.84±0.34	Light pale yellow	Compact
	2.00	67.53±0.20	Pale yellow	Compact
2,4-D + NAA	1.5+1.0	91.11±0.12	Pale yellow	Compact
	2.0+1.0	95.22±0.19	Light pale yellow	Compact
	2.5+1.0	93.44±0.31	Pale yellow	Compact
	3.0+1.0	86.89±0.23	Pale yellow	Compact
2,4-D + BAP	1.50 + 0.10	43.12±0.18	Brownish	Friable
	2.00 + 0.10	64.75±0.34	Brownish	Friable
	2.00 + 0.20	74.32±0.52	Brown	Friable
	2.50 + 0.50	66.56±0.37	Brown	Friable
NAA +BAP	1.00 + 0.20	42.98±0.17	Bright yellow	Compact
	1.50 + 0.50	73.78±0.29	Bright yellow	Compact
	1.50 + 1.00	86.65±0.44	Bright yellow	Compact
	2.00 + 1.50	64.54±0.27	Bright yellow	Compact

Table 2. Effects of different hormonal combinations (2, 4-D, NAA, 2, 4-D + NAA, 2, 4-D +BAP and NAA + BAP) on development of embryogenic calli after 12 days of sub culture of BRRI dhan56.

PGRs	PGR concentrations (mg/l)	Percentage of embryogenic calli	Color of calli	Morphological features of calli
2,4-D	1.5	80.67±0.12	Pale yellow	Compact
	2.0	85.45±0.15	Pale yellow	Compact
	2.5	82.66±0.32	Pale yellow	Compact
	3.0	77.59±0.09	Pale yellow	Compact
NAA	0.50	41.86±0.07	Pale yellow	Compact
	1.00	59.43±0.34	Pale yellow	Compact
	1.50	66.53±0.51	Light pale yellow	Compact
	2.00	57.78±0.32	Pale yellow	Compact
2,4-D + NAA	1.5+1.0	82.11±0.31	Pale yellow	Compact
	2.0+1.0	88.22±0.50	Light pale yellow	Compact
	2.5+1.0	83.44±0.21	Pale yellow	Compact
	3.0+1.0	77.89±0.09	Pale yellow	Compact
2,4-D + BAP	1.50 + 0.10	43.90±0.14	Brownish	Friable
	2.00 + 0.10	62.52±0.21	Brownish	Friable
	2.00 + 0.20	71.32±0.44	Brown	Friable
	2.50 + 0.50	66.67±0.32	Brown	Friable
NAA +BAP	1.00 + 0.20	39.96±0.08	Bright yellow	Compact
	1.50 + 0.50	71.07±0.19	Bright yellow	Compact
	1.50 + 1.00	84.32±0.33	Bright yellow	Compact
	2.00 + 1.50	71.08±0.21	Bright yellow	Compact

Genetic transformation of rice callus

In our present investigation, *Agrobacterium* strain LBA4404 harboring the plasmid pBI121 contains kanamycin resistance gene (npt*II*) as the selectable marker gene. Kanamycin was tested as a selective agent for transgenic rice BRRI dhan56. Infected calli from mature seeds of BRRI dhan56 were cultured on shoot induction medium containing various concentrations (50, 75, 100, 125 and 150 mg/l) of kanamycin. Shoot regeneration was greatly inhibited by the increased concentration of kanamycin (Figure 2). The highest response was 32.0% of the explants regenerated shoots in the presence of 100 mg/l kanamycin and there was no shoot regeneration with 150 mg/l or greater concentrations of kanamycin. The effects of different incubation periods (15, 20, 25, 30 and 35 min) with the *Agrobacterium* strain on the calli were studied at a constant optical density (OD_{600} of ~0.9). In every case, 10 calli were infected and the highest 32% *GUS* positive result was found with 25 min incubation period. On the other hand, the lowest 10% *GUS* positive result was found with 15 min incubation period (Figure 3). Optical density (OD) of *Agrobacterium* strain is an important factor for genetic transformation in rice. In this study, five

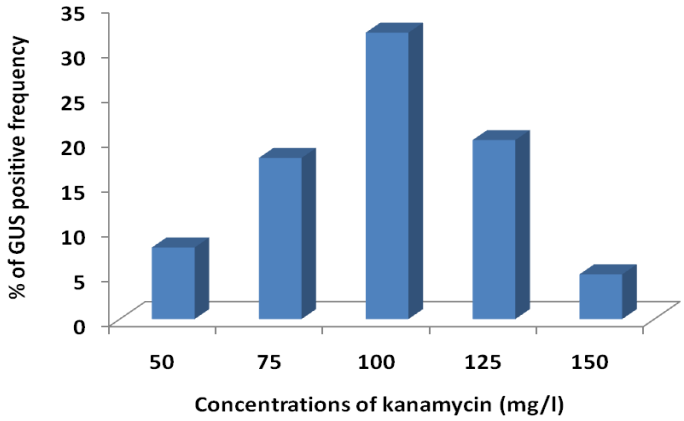

Figure 2. Effect of kanamycin concentrations (50, 75, 100, 125 and 150 mg/l) on shoot regeneration from calli of mature seed of elite rice cultivar BRRI dhan56.

different optical density levels were tested viz., 0.6, 0.8, 0.9, 1.0 and 1.1 (Figure 4). The highest number of GUS expressing calli was observed at OD 0.8 to 0.9. It was clearly confirmed that the bacterial strain LBA4404 (pBI121) showed highest peak of performance in lower

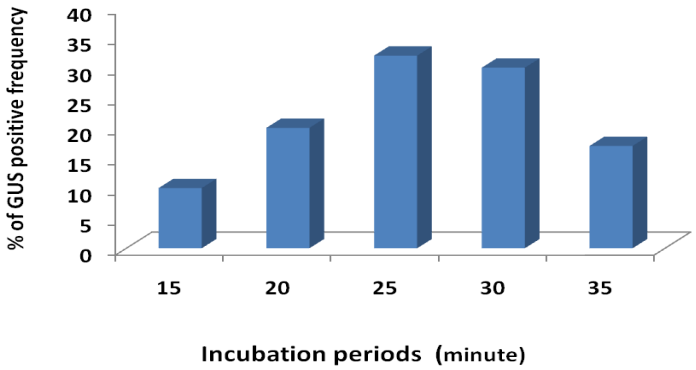

Figure 3. Transformation efficiency (%) of the 3 week old calli of BRRI dhan56 incubation time with *Agrobacterium* strain LBA 4404 which harboring the plasmid pIG121 at 15, 20, 25, 30 and 35 min.

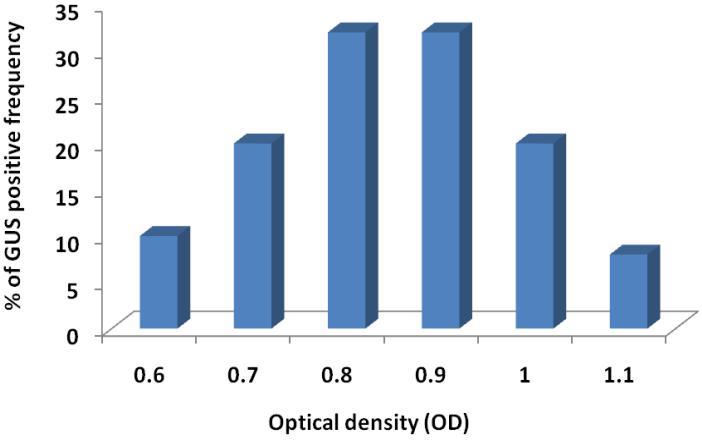

Figure 4. Transformation efficiency (%) of the 3 weeks old calli of BRRI dhan29 co-cultivated with *Agrobacterium* strain LBA 4404 which harboring the plasmid pIG121 at 0.6, 0.8, 0.9, 1.0 and 1.1 OD600.

range of OD and it gradually decreases with the increase of OD taken.

Duration of co-cultivation, the temperature of incubation, with *Agrobacterium*, concentration and the composition of bacteriostatic agent and duration of selection and concentration of antibiotic selection marker are the other important factors reported which affect *Agrobacterium* mediated transformation in rice (Okkels and Pederson, 1998; Katiyar et al., 1999). To find the optimum conditions for co-cultivation, different concentration of acetosyringone and the duration of co-cultivation were tested very carefully. The frequency of kanamycin resistant calli obtained following each variation in co-cultivation conditions was taken as the transformation frequency. For optimum transformation, the presence of 100 µM acetosyringine in co-cultivation medium, 500 mg/L cefotaxime to inhibit *Agrobacterium* growth and co-cultivation for three days were found to be the most suitable.

Stable integration of foreign gene and confirmation

After inoculation with *Agrobacterium,* the explants were co-cultivated for 3 days on normal MS media. The explants were then sub-cultured on regeneration medium (BAP 2.0 mg/l + 1.0 mg/l NAA + 1.5 mg/l KIN) containing carbenicillin. With 2 to 3 weeks of culture, the calli developed shoot buds (Figure 5A and B) and were sub-cultured on the same medium for shoot development (Figure 5C). After proliferation, shoot were transferred on selection medium with kanamycin and the same hormonal combination (Figure 5D) first cycle and second cycle selection. At the same time control plants were subsequently cultured on selection medium first cycle and second cycle selection and 90% shoots died after 21 days. A bacteriostatic agent namely cefotaxime was used to inhibit *Agrobacterium* growth after co-cultivation. It has been recorded that the use of high concentration of bacteriostatic agents may reduce the regenerability of the calli as they structurally resemble auxins. Further, in combination with other callus inducing hormones such as 2, 4 D may cause loss in regeneration potential (Lin et al., 1995; Okkels and Pederson, 1998). Regenerated shoots harvested from selection medium and transfer in the normal MS medium for vigorous root development (Figure 5E) before micro plant transfer in pot for establishment. After six days, a well-developed root system was observed in the plantlets. Then the plantlets transferred to soil survived under the normal environmental conditions and grew to maturity. With the purpose of confirm the presence of the transgene, calli were subjected to histochemical staining. GUS assay was performed according to Jefferson (1987) in two stages, first, After 3 days of co-cultivation in calli and second the leaves and roots from putative plants were taken and incubated in X-*gluc* buffer. Indigo blue coloration was observed in calli (Figure 6A), section of calli (Figure 6B), leaves and root (Figure 6C and 6D). Appearance of blue color following overnight incubation at 37°C with GUS assay revealed the presence of the transgene. GUS gene was amplified in transformed plant DNA and plasmid DNA where forward and reverse primer amplified 880 bp *GUS* gene segment (Figure 7). Compared to the non-transformed calli with regeneration frequency of 96% and transformed calli showed very low regeneration frequency (32%). A researcher (Kumar et al., 2005) recorded a transformation efficiency of 4.6 to 5.5% and 6.4 to 7.3% for two recalcitrant elite *Indica* rice cultivars, which were lower transformation frequencies than these results.

Conclusion

In conclusion, we have established rapid multiple shoot induction and efficient plant regeneration method from seeds elite indica rice cultivar BRRI dhan56 in a genotype independent manner. The combination of 2, 4-

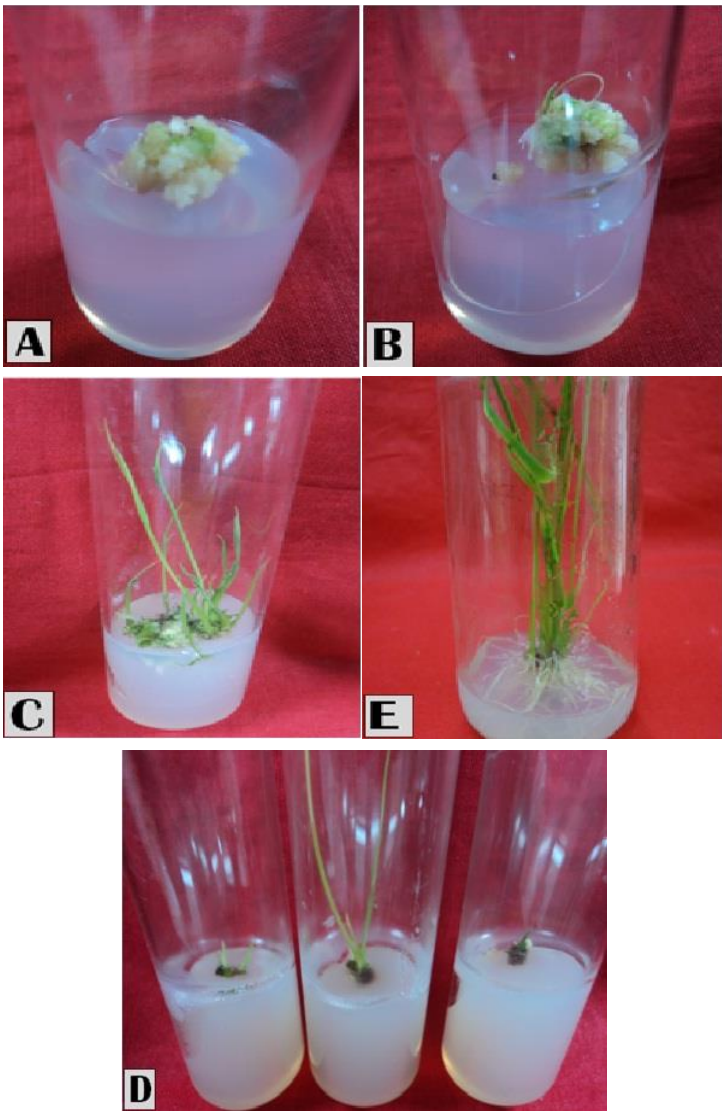

Figure 5. (A) Shoot bud development, **(B)** shoot initiation, **(C)** Shoot elongation, from transform calli in regeneration medium (BAP 2.0 + NAA 1.0 + KIN 1.5) containing 100 mg/l carbencillin. First cycle selection: **(D)** Shoots of transformed calli and **(E)** control in selection medium with 75 mg/l kanamycin.

D + NAA (2.0+1.0 mg/l) was shown to enhance the highest callus induction, where 95.22% explants produced calli in BRRI dhan56. Compared to the non-transformed calli with regeneration frequency of 96% and transformed calli showed very low regeneration frequency (32%). For optimum transformation, the presence of 100 µM acetosyringine in co-cultivation medium, 500 mg/L cefotaxime to inhibit *Agrobacterium* growth, OD_{600} 0.8-0.9 of *Agrobacterium* strain and co-cultivation for 3 days were found to be the most suitable. The method described was simple, inexpensive and does not require any advanced equipment. This study would be an effective tool for crop improvement and gene-function studies on the model monocot plant rice.

Conflict of interests

The authors did not declare any conflict of interest.

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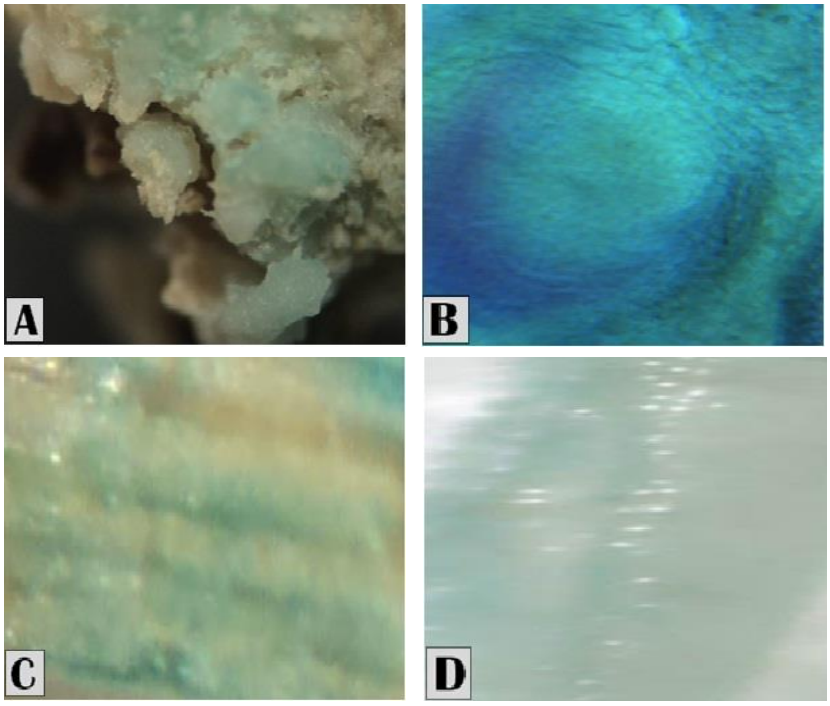

Figure 6. (A) Expression in transformed callus and **(B)** section of transform callus after 3 days of co-cultivation, **(C)** *GUS* expression in leaf segment and **(D)** root segment of putative plant.

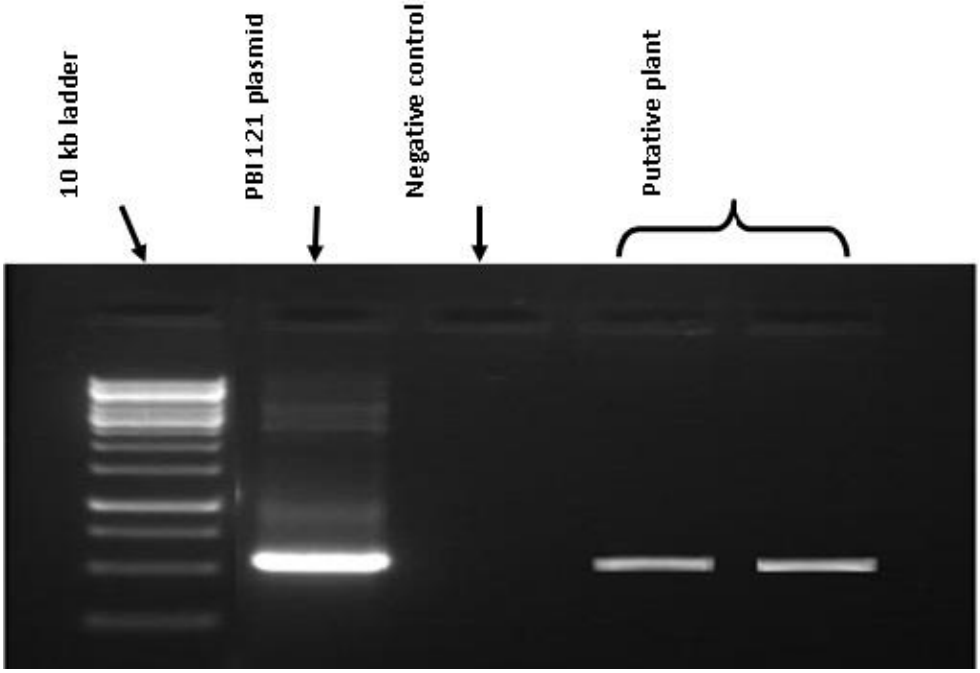

Figure 7. Confirmation of *GUS* gene through PCR analysis.

grateful to DNA and Chromosome Research Laboratory, University of Rajshahi, Rajshahi-6205, Bangladesh.

Abbreviations: GUS, β-Glucuronidase; **PCR,** polymerase chain reaction; **MS,** Murashige and Skoog; **2,**

4-D, 2,4-dichlorophenoxyacetic acid; **MCI,** callus induction medium; **OD,** optical density; **NAA,** 1-naphthaleneacetic acid; **BAP,** 6-benzylaminopurine.

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Full Length Research Paper

Impact of organic and inorganic fertilizers on growth, fruit yield, nutritional and lycopene contents of three varieties of tomato (*Lycopersicon esculentum* (L.) Mill) in Ogbomoso, Nigeria

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Field experiments were carried out to assess the impact of 100% NPK fertilizer recommendation (300 kg NPK 20:10:10), 100% Tithonia compost (TC), 75% NPK + 25% TC, 50% NPK + 50% TC, 25% NPK + 75% TC and control (non-fertilized plant) on the growth, fruit yield, nutritional and lycopene contents of three tomato varieties (Raoma VF; Ogbomoso local and Califonia wonder). The experiment was a split plot fitted into a randomized complete block design replicated three times. Data were collected on growth and reproductive parameters, fruit and seed attributes, and fruit proximate and nutritional contents. Data collected were subjected to analysis of variance to determine significant means. Significant varietal differences were observed among the tomato varieties in terms of growth, fruit yield and nutritional attributes. The plant dry matter yield was highest in Ogbomoso local and least in Roma VF. Fruit yield obtained with Ogbomoso local was 45 and 56% higher than what was obtained for Roma VF and Califonia wonder, respectively. In terms of vitamin C content, Roma VF fertilized with 50% NPK + 50% TC gave the highest value which is 23 to 67% higher than values obtained from the other treatment combinations. Again, irrespective of variety, organically grown tomato contains higher content of lycopene. It was also observed that the higher the proportion of TC compost in the treatments, the better the lycopene content. It could be concluded that the use of organic fertilizer has potential in improving the growth, fruit yield and nutritional contents of any of the three tomato varieties studied.

Key words: *Lycopersicon esculentum*, compost, inorganic fertilizers, fruit yield, nutritional quality, lycopene content.

INTRODUCTION

Tomato (*Lycopersicon esculentum* (L.) Mill) of the family Solanaceae, is one of the most important vegetable crops in the world. It is consumed fresh and as paste in all parts of the world (Alofe and Somade, 1982). Tomato plays a

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Abbreviations: TC, Tithonia compost; **WAT,** week after transplanting; **TSS,** total soluble solid.

vital role in the improvement of the diet of mankind. The fruit is adapted to various culinary uses either in the fresh form in salad or as puree in gravies, stew and soups, for the diet of the diverse cultures of the world. More than 90% of the vitamin C in human diets is supplied by fruits and vegetables (of which tomato is the most important) (Vallejo et al., 2002). Tomato fruits contain high amount of ascorbic acid and lycopene (Tindall, 1983). Lycopene, an antioxidant, is the pigment that imparts red color to some fruits, most notably tomato and watermelon. It is also a highly efficient oxygen radical scavenger and has been implicated in human health as providing protection against cardiovascular disease and some cancers, particularly that of the prostate.

Many pre- and post harvest factors influence the phytochemical contents of horticultural crops. Large genotypic variation in vitamin content was reviewed by Kurilich et al. (1999) and Vallejo et al. (2002). Other preharvest factors include climatic conditions and cultural practices (Howard et al., 1999; Lisiewska and Kmiecik, 1996; Jeffery et al., 2003). Among the cultural practices, fertilizers and soil fertility can influence the level of functional food components in crops.

The society has been increasingly concerned about environmental damage caused by agricultural activities, especially with regard to health hazards resulting from the use of agrochemicals (Van der Berge et al., 2000). Many alternative cropping systems have been developed and among them, organic agriculture has been established and certified in many countries (Adediran et al., 2003). Organic agriculture is characterized by the absence of synthetic fertilizer and pesticides in addition to the frequent utilization of organic fertilizers as sources of crop nutrients (Van der Berge et al., 2000; Adediran et al., 2003). The acceptance of crop produced can be influenced by the source of nutrients involved in its production. In the recent past, some studies have been conducted to elucidate the beneficial effects of adding crop residue compost into the soil. The practice improves soil physical, chemical and biological activities as well as improving crop yields and nutritional values (Manna et al., 1999; Akanbi and Togun, 2002; Adediran et al., 2003; Maharishnan et al., 2004; Ghosh et al., 2004; Ashutosh et al., 2006). The supply of organic materials on farms, even with the use of farm yard manure and or compost from crop residues, will likely be insufficient to overcome soil nutrient deficiency. The integration of small amount of inorganic fertilizer with the organic materials available on farms offers a strategy to meet the nutrient requirements of crops. It minimizes nutrient leaching, particularly in poor sandy soil and subsequent groundwater contamination (Manna et al., 1999). This maximizes the use of available organic resources and minimizes the use of costly purchased mineral fertilizers (Manral and Saxena, 2003; Ghosh et al., 2004). Crop growth, yield and product quality in relation to application of agro waste compost has been widely reported (Togun et al., 2003). For instance, high fruit yield due to compost application were reported on tomatoes with combine application of 2 t/ha compost and 30 kg N/ha. In most of these studies, compost application was observed to have positive effects which aid crop growth and development thereby improving the crop phytonutritional components (Togun et al., 2003).

Despite many investigations in the area of nutrition, knowledge on how organic fertilizers in combination with genotypic variation influences physical and phytochemical contents of tomato fruit is inadequate. This study assessed variability of plant growth, fruit physical and nutritional qualities among three commonly cultivated tomato varieties in Nigeria as influenced by organic and inorganic fertilizers.

MATERIALS AND METHODS

The studies were conducted at the Teaching and Research Farm, Ladoke Akintola University of Technology, Ogbomoso in 2005 and 2007. Ladoke Akintola University of Technology Ogbomoso is located on latitude 8° 10'N and longitude 4° 10' and the altitude is about 420 m above sea level. Ogbomoso lies in the transitional zone between forest and guinea savanna. It has a bimodal rainfall pattern with peaks in July and September, with a short rainfall break in August. The bimodal rainfall of the area is between 150 and 125 mm. The temperature regime is high all the year round. The mean minimum temperature is 28°C and the maximum temperature is 33°C with a high relative humidity of about 74% all year round except in January when the dry wind blow from the north.

The vegetation of the area is composed of weed species like wild sunflower (*Tithonia diversifolia), Tridax procumbens, Talinum triangulare, Imperata cylindrica*, Sedges sp. and guinea grass mixed with scattered shrubs and trees notably locust bean trees (*Parkia biblobosa*). The experimental site has been under cassava cropping for 2 years before being cleared for use.

Two field experiments were conducted in early 2005 and 2007 cropping seasons, using a split plot in randomized complete block design replicated three times. The factors tested were three tomato varieties (Califonia wonder, Ogbomoso local and Roma VF) and six fertilizer types {0% NPK or Tithonia compost (TC); 100% NPK fertilizer recommendation (300 kg NPK 20:10:10); 100% TC; 75% NPK + 25% TC; 50% NPK + 50% TC and 25% NPK + 75% TC}. The tomato variety formed the main plot, while the six fertilizer types formed the subplot factors. The three varieties form the bulk of tomato cultivars being cultivated and adapted to most agro ecological zones in Nigeria. The Roma VF and Califonia wonder are determinate types, while Ogbomoso local is indeterminate one. The seeds of Roma VF and Califonia wonder were obtained from the Genetic and Seed Resources Unit of National Institute for Horticultural Research and Training, Ibadan, while that of Ogbomoso local was obtained from 'Arada' open market, Ogbomoso, all in Nigeria. The 100% NPK treatment was chosen based on general fertilizer recommendation for tomato plant in south west Nigeria (FPDD, 1990), while quantity of Tithonia compost (TC) applied in the 100% TC treatment was arrived at by considering the tomato N nutrient recommendation (60 kg N/ha) and the N content of TC (2.4% N). Each replicate contained all the 18 treatments combinations. The crop was spaced out 50 x 50 cm with 1 plant per stand. A subplot measured 2.5 x 2.5 m (6.25 m^2) and contained six rows of crop and each row had 6 plants making 36 plants per subplot. Each main plot measured 20 x 2.5 m (50 m^2) and gaps of 1 m separated all the sub and main plots. A replicate was 20 x 9.5 m (190 m^2) in dimension and adjacent replicates were separated by gaps of 2 m. The total experimental area was 64.0 x

9.5 m (608.0.m^2).

The seeds of the 3 tomato cultivars were sown in a box containing 1:3 top soil: compost proportion by weight (Akanbi et al., 2002). The seedlings were allowed to grow for a period of 28 days. At transplanting, healthy seedlings of each cultivar were selected and transplanted into well prepared beds on 20th of April, and 26th of April, for 2005 and 2007 experiment, respectively. Supplying of vacant stand was done a week after transplanting (WAT). Compost treatments were applied at transplanting, while NPK mineral fertilizer treatments were applied two WAT. Manual weeding was done thrice starting from 2 WAT and repeated every 3 weeks interval by hoeing. Insect pests were controlled by spraying the crops with Karate at the rate of 40 ml / 20 L water at two weeks interval starting from 2 WAT. The crops were individually staked with 1 m stake between 4 and 5 WAT.

Six plants were randomly tagged per plot for data collection. The growth parameters measured were per plant number of functional leaves and offshoots, plant stem girth and dry matter yield, while the reproductive and fruit/seed traits considered were number of flowers and fruit/plant, percentage fruit sets, fruit length and diameter, total fruit number and weight, number and weight of seeds / fruit and total fruit yield.

For determination of fruit phytochemical contents at full ripening, 12 (for 2005 experiment) and 8 (for 2007) fruit samples of uniform ripening were randomly selected per subplot and analysed for pH, total soluble solid (TSS), moisture content, crude protein, crude fibre, ether extract, vitamin C and lycopene contents. Proximate compositions were determined using AOAC (1984) method. The fruits were first homogenized in Wiley Micro-Hammer Stainless mill. The pH of the homogenized pulp was determined using pH meter. The total pulp N was determined by a semi micro-kjeldahl procedure (Bremner, 1965; Ulger et al., 1997) and fruit protein was calculated from the Kjeldahl nitrogen using the conversion factor 6.25. Crude fibre content was estimated from the loss in weight of the crucible and its content on ignition. 50 g homogenized pulp is digested in 1.25% tetra-oxo-sulphate (IV) acid and 1.25% sodium hydroxide. The digest was put in crucible and transferred into a muffle furnace at 550 for 3½ h. The weight difference expressed as a percentage of the fresh weight constitutes the percent crude fibre. Ether extract was estimated by exhaustively extracting a known weight of sample with petroleum ether (BP 60°C) using a Tecator Soxhlet apparatus. The TSS was determined by using the hand refractometer (Adebooye et al., 2006) and vitamin C content was determined by using the indolphenol dye method (Jagdish et al., 2007). The lycopene content was determined by grinding 20 ml of the homogenized pulp in 25 ml acetone and 20 ml hexane and the absorbance was read at 50 l nm using a colourimeter. Mineral elements were estimated using the AOAC (1984) method. The atomic absorption spectrometer was used to determine Ca, K and Fe. Phosphorus (P) was determined using the colorimetric molybdenum-blue procedure (Murphy and Riley, 1962).

The analysis of variance was performed on the data following procedure of Gomez and Gomez (1984) and significant means were compared using Duncan's multiple range test ($P \leq 0.05$). Correlations were run among parameters to test their association.

RESULTS AND DISCUSSION

Variability of tomato vegetative parameters in response to applied fertilizer types are presented in Table 1. Number of leaves and offshoot/plant, stem girth and dry matter yield were all significantly ($P \leq 0.05$) affected by tomato variety and fertilizer types. Ogbomoso local had the highest number of leaves/plant; this is followed by Roma VF, while Califonai wonder had the least. Combined application of 25% NPK + 75% TC produced highest number of leaves which is significantly better than what was observed with other treatments with the exception of 100% NPK treatment. Among the treatments that contained compost, there was an improvement in the parameter as the compost content increased. Variability of stem girth in relation to tomato variety and fertilizer combination was significant. Roma VF had the most robust stem, while Califonia wonder had the least. Among the fertilizer combinations, the use of 100% TC gave the best stem girth which was not significantly different from 75% NPK + 25% TC and 25% NPK + 75% TC treatments. The Roma VF fertilized with 100% TC gave the best stem girth. Number of offshoot /plant varied significantly in line with what was observed with number of leaves/plant. However, observation made on this parameter in response to applied fertilizer combination was not the same. The offshoot of tomato plant nourished with 100% TC was significantly better than values obtained for all other treatments. Dry matter production varied significantly across the varieties and fertilizer combination. The main effect of variety showed that Ogbomoso local accumulate the highest dry matter (38.5 g/plant) which was 40 and 53% higher than dry matter obtained for Califonia wonder and Roma VF, respectively. The dry matter content of tomato fertilized with 50% NPK + 50% TC was significantly higher than the ones accumulated using other fertilizer types, while Ogbomoso local fertilized with 50% NPK + 50% TC gave the highest interactive effect (80 g/plant). Variability of vegetative development of the 3 tomato varieties used could be attributed to differences in genetic makeup and variation in the source and quantity of nutrients used. Large genotypic variation in vegetative and vitamin content of some crops had been reported by Kurilich et al. (1999) and Vallejo et al. (2002). Ogbomoso local, being indeterminate one, produce more vegetative parameters over other varieties. This is in line with the report of Vallejo et al. (2002). Application of 100% NPK and 25% NPK + 75% TC gave the best vegetative development. Availability of optimum amount of nutrients for plant use is to enable plants to exhibit their optimum potential. The use of 100% NPK or 25% NPK + 75% TC could be regarded as optimum rate for tomato vegetative development. These rates contained sufficient amount of needed nutrients hence plants nourished with them have better performance. Such observation on tomato was reported by Akanbi and Togun (2002).

The interactive effects of fertilizer types and tomato variety on number of flowers/plant and percent fruit sets are presented in Figure 1. Irrespective of fertilizer types, Ogbomoso local produced the highest number of flowers, followed by Roma VF, while Califonia wonder had the least. In term of percentage fruit set, Califonia wonder had the highest followed by Roma VF, while Ogbomoso local had the least. This observation could be attributed to higher leaves and offshoot produced by the Ogbomoso

Table 1. Effect of fertilizer types and variety on vegetative growth of tomato at 8 weeks after transplanting.

Fertilizer type	Tomato variety			Mean
	Califonia wonder	Ogbomoso local	Roma VF	
Number of leaves/plant				
0 kg	5.8	22.1	8.2	12.0^{d}
100% NPK	5.2	23.0	17.2	15.1^{ab}
100% TC	10.6	22.8	10.2	14.5^{c}
75% NPK +25% TC	7.0	21.6	10.0	12.9^{c}
50% NPK + 50% TC	6.2	18.3	19.8	14.8^{c}
25% NPK + 75% TC	8.0	27.3	19.6	18.3^{a}
Mean	7.1^{c}	22.5^{a}	14.2^{b}	
Stem girth (cm)				
0 kg	1.8	2.75	2.9	2.5^{c}
100% NPK	2.8	3.1	3.3	3.0^{b}
100% TC	2.7	2.7	4.1	3.2^{a}
75% NPK + 25% TC	2.8	3.2	3.2	3.1^{ab}
50% NPK + 50% TC	2.5	3.1	3.1	2.9^{b}
25% NPK + 75% TC	2.9	3.1	3.2	3.1^{ab}
Mean	2.6^{c}	2.9^{b}	3.3^{a}	
Number of offshoot/plant				
0 kg	2.7	9.0	4.5	5.4^{e}
100% NPK	3.5	7.5	7.5	6.2^{d}
100% TC	9.0	9.2	9.2	9.2^{a}
75% NPK + 25% TC	4.7	10.3	6.5	7.2^{c}
50% NPK + 50% TC	5.5	9.3	7.2	7.3^{c}
25% NPK + 75% TC	7.5	11.5	5.7	8.3^{b}
Mean	5.5^{c}	9.5^{a}	6.8^{b}	
Dry matter (g/plant)				
0 kg	15.5	57.5	9.6	27.5^{b}
100% NPK	22.1	16.2	14.8	17.7^{d}
100% TC	21.5	37.0	11.5	23.3^{c}
75% NPK + 25% TC	27.5	19.0	19.5	22.0^{c}
50% NPK + 50% TC	20.6	80.1	39.1	46.6^{a}
25% NPK + 75% TC	14.7	21.8	13.8	15.9^{e}
Mean	20.3^{b}	38.5^{a}	18.0^{c}	

Means followed by the same letter along the column are statistically similar (DMRT, 5%).

local. The primary and secondary vines (offshoot) provided axis and loci for flower production. However, higher number of flowers may not translate into higher number of fruits. This account for the reason why Ogbomoso local did not recorded the highest percentage fruit set. The implication of this is that plant with higher potential for flower formation must have efficient accumulation of photoshynthentates in order to support the flowers. This tendency is low in Ogbomoso local, hence many of the flowers produced were aborted. This culminated into having fewer number of fruits when compared to other varieties. This observation is supported by reports of Manna et al. (1999) and Adediran et al. (2003).

The main and interactive effects of fertilizer types and tomato variety on tomato fruit and seed parameters are presented in Table 2. Fruit length and diameter as well as per fruit number and weight of seeds were all significantly affected by the applied treatments. Fruit length varied from 8.2 cm in Califonia wonder to 7.4 cm in Roma VF.

Application of 75% NPK + 25% TC gave the longest fruit. This was significantly higher than values obtained with other treatments with the exception of 100% NPK treatment. In case of fruit diameter, Ogbomoso had the widest, while Roma VF had the least. Application of

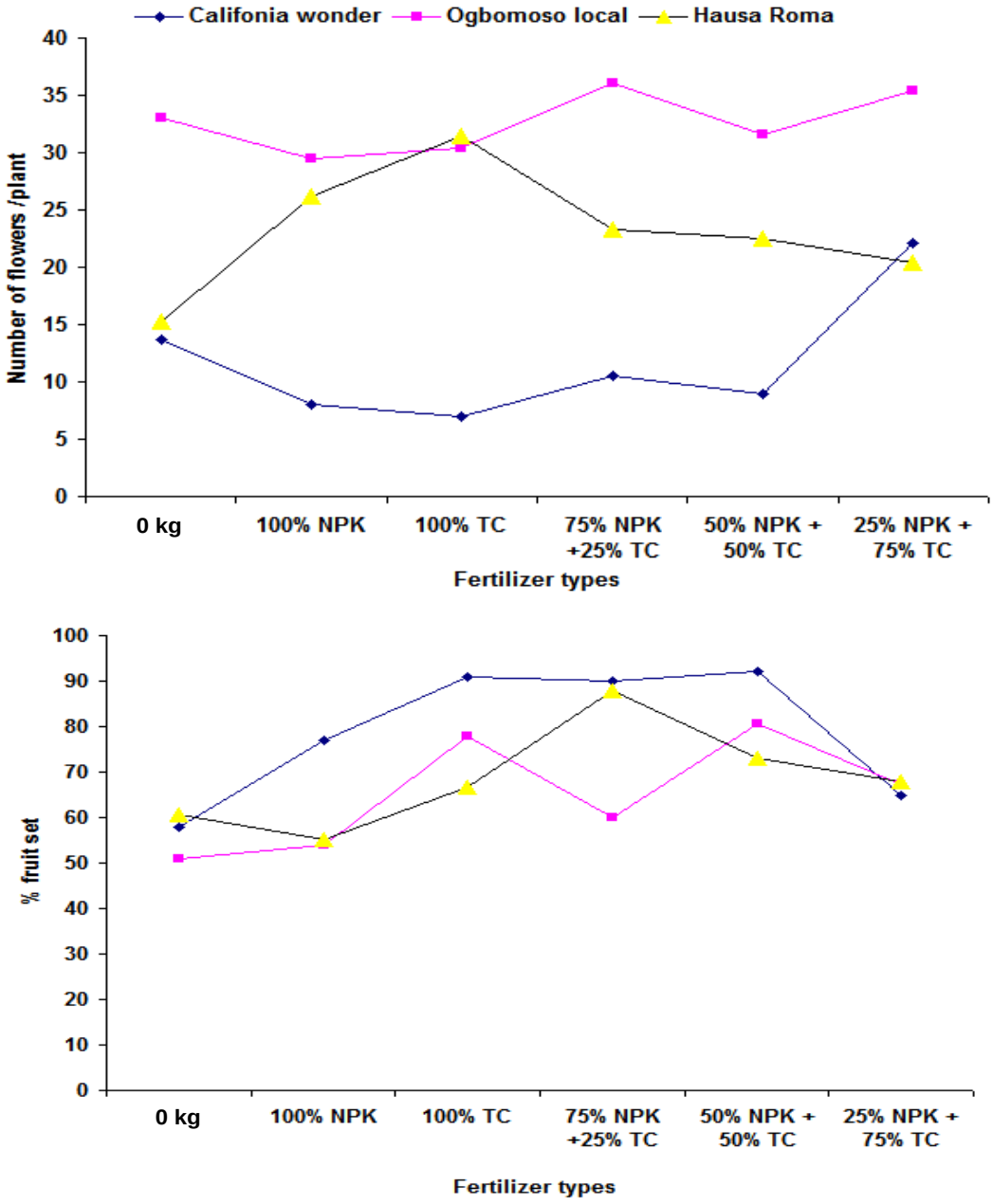

Figure 1. Effect of fertilizer types on number of flowers/plant and percent fruit sets of three varieties of tomato.

100% NPK gave the widest fruit diameter (15.9 cm), while the least (13.8 cm) was obtained with the use of 100% TC.

Per fruit number and weight of seeds varied significantly ($P \leq 0.05$). Ogbomoso local had the highest number of seeds per fruit. The value obtained with this variety was 34 and 48% higher than what was observed with Califonia wonder and Roma VF, respectively. Application of 100% TC had the highest number of seeds/fruit and it was significantly higher than other treatments. For interactive effects, Ogbomoso local that received 100% TC produced fruit that has the highest number of seeds. As much as seed weight /fruit is concern, Ogbomoso local had the highest (0.36 g /fruit) followed by 0.24 and 0.16 g/fruit obtained from Roma VF and Califonia wonder, respectively. Again, fertilizer types had significant effect on seed weight/fruit. Irrespective of variety, non fertilized plants had the heaviest seed weight, while 100% NPK treatment had the least. It is worthy to note that among the treatments that contained TC, the higher the compost content, the heavier the seeds weight per fruit.

Table 2. Effect of fertilizer types and variety on fruit and seed parameters of tomato.

Fertilizer type	Tomato variety			Mean
	Califonia wonder	Ogbomoso local	Roma VF	
Fruit length (cm)				
0 kg	6.4	8.2	8.3	7.6^{c}
100% NPK	8.3	9.5	7.4	8.4^{a}
100% TC	8.5	6.3	7.4	7.4^{d}
75% NPK + 25% TC	10.1	8.2	7.3	8.5^{a}
50% NPK + 50% TC	7.4	7.1	6.7	7.1^{e}
25% NPK + 75% TC	8.2	8.0	7.3	7.8^{b}
Mean	8.2^{a}	7.9^{b}	7.4^{c}	
Fruit diameter				
0 kg	14.3	19.8	11.9	15.4^{b}
100% NPK	15.0	19.8	12.9	15.9^{a}
100% TC	16.2	17.7	7.4	13.8^{c}
75% NPK + 25% TC	16.0	18.5	7.3	13.9^{c}
50% NPK + 50% TC	15.2	19.2	12.2	15.5^{ab}
25% NPK + 75% TC	14.5	14.4	12.5	13.9^{c}
Mean	15.2^{b}	18.2^{a}	10.7^{c}	
Number of seeds/fruit				
0 kg	27.3	70.6	62.4	53.4^{d}
100% NPK	25.0	72.3	47.4	48.2^{e}
100% TC	60.3	106.6	64.4	77.1^{a}
75% NPK + 25% TC	41.0	73.1	65.7	59.9^{b}
50% NPK + 50% TC	33.0	71.0	62.0	55.3^{c}
25% NPK + 75% TC	41.0	76.0	62.0	59.7^{b}
Mean	37.9^{b}	78.3^{a}	60.7^{a}	
Seed weight/fruit (g)				
0 kg	0.14	0.53	0.24	0.32^{a}
100% NPK	0.18	0.12	0.17	0.13^{e}
100% TC	0.22	0.14	0.25	0.23^{d}
75% NPK + 25% TC	0.15	0.46	0.24	0.25^{c}
50% NPK + 50% TC	0.15	0.53	0.16	0.34^{b}
25% NPK + 75% TC	0.14	0.38	0.34	0.23^{c}
Mean	0.16^{c}	0.36^{a}	0.24^{b}	

Means followed by the same letter along the column are statistically similar (DMRT, 5%).

Responses of the tomato fruit yield in relation to applied treatments are presented in Figure 2. Significant varietal differences existed in fruit yield of the three tomato varieties tested in this study. Ogbomoso local produced highest fruit yield (20.9 t/ha), followed by Roma VF (15.3 t/ha) and the least was obtained from Califonia wonder (11.3 t/ha). Applied fertilizer types significantly influenced the fruit yield. Application of 100% TC produced the highest fruit yield, while the least was obtained from nonfertilized control treatment. The Ogbomoso local that received 100% TC, produced the highest interactive effect.

Table 3 contained data collected on effects of fertilizer types on elemental composition of fruit of three varieties of tomato. The varietal differences, fertilizer types and their interactions are significant on tomato fruit elemental compositions. The P content varied from 21.9 g/100 g in Roma VF to 18.5 g/100 g in Califonia wonder. The fruits harvested from tomato plants nourished with 100% TC contained the highest P. The value obtained with this treatment was significantly higher than what was obtained with other treatments with the exception of 25% NPK + 75% TC. The K, Ca and Mg contents of Ogbomoso local and Roma VF were similar and

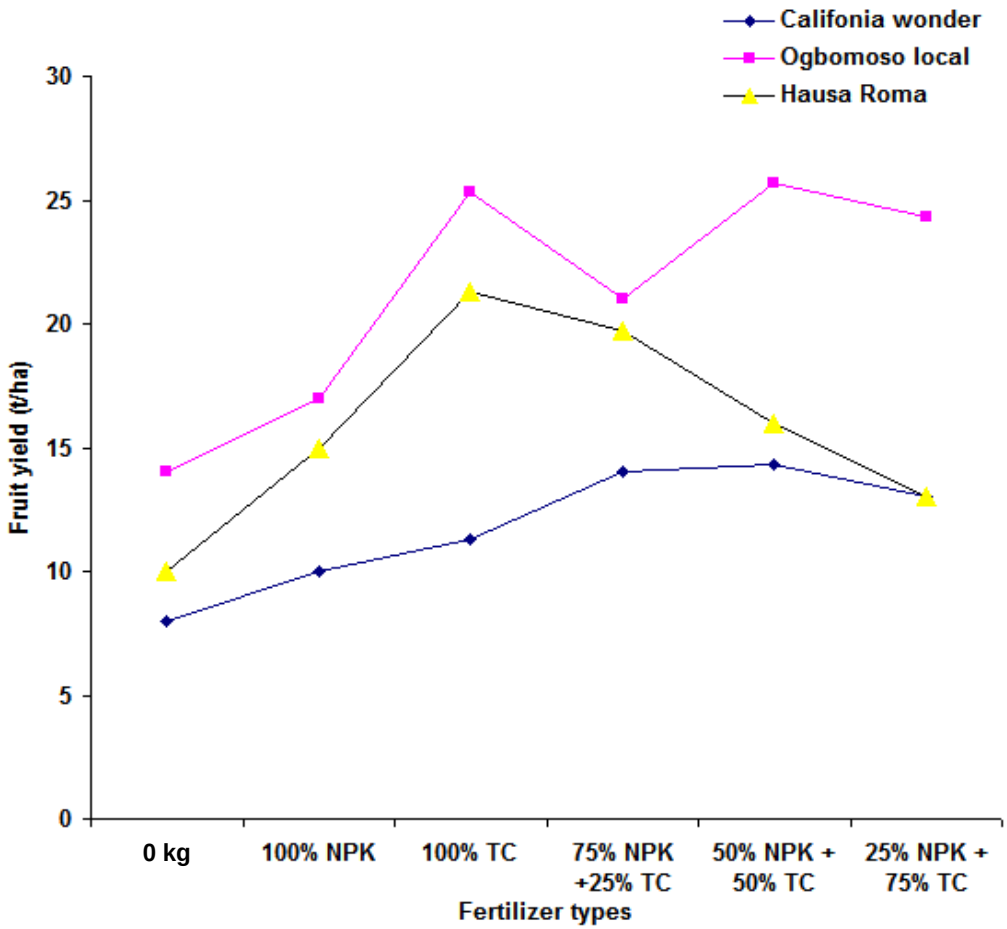

Figure 2. Effect of fertilizer types on fruit yield of three varieties of tomato.

significantly higher than that of Califonia wonder. The K and Ca contents of 25% NPK + 75% TC treatment were higher than values obtained from other treatments. Magnesium content of plants fertilized with 100% TC (19.9 g/100 g) was the highest, while that of 75% NPK + 25% TC (16.3 g/100 g) was the least. Tomato fruit proximate composition in response to varietal differences and fertilizer types are shown in Table 4. All the parameters considered were significantly influenced by variety, fertilizer types and their interaction.

The fruit pH ranges from 5.17 in Ogbomoso x 100% TC to 5.82 in Roma VF x 50% NPK + 50% TC. The crude protein content of Ogbomoso local (2.21 g/100 g) compared favourably with 1.81 g/100 g obtained from Califonia wonder. Among the two way interactions, Ogbomoso local variety fertilized with 100% TC had the highest crude protein, while the least was obtained from Roma VF fertilized with 100% TC. The crude fibre of Ogbomoso local was higher than those of the other two varieties. Non fertilized Ogbomoso local variety had the highest crude fibre contents. Ether extracts ranges from 1.27 g/100 g in non fertilized Ogbomoso local variety to 0.36 in Califonia wonder x 75% NPK + 25% TC. On the average, Califonia wonder contained higher amount of total soluble solid, this is followed by Roma VF, while Ogbomoso local had the least.

Effect of variety, fertilizer types and their interactions are significant on tomato fruit vitamin C and lycopene contents. The vitamin C content of Roma VF was the highest (30.1 mg /100 g), while the least (17.1 mg /100 g) was obtained from Ogbomoso local. Among the fertilizer types used, 75% NPK + 25% TC and 50% NPK + 50% TC had similar vitamin C contents, and their values were significantly higher than vitamin C contents of other treatments. The two way interactive effects of variety and fertilizer types revealed that Roma VF nourished with 75% NPK + 25% TC or 50% NPK + 50% TC had the highest vitamin C content.

Results in Table 5 showed that fertilizer types had significant effects on fruit lycopene contents of three varieties of tomato used. As with the vitamin C, Roma VF variety had the highest lycopene content (0.59 mg/100 g) which was similar to 0.52 mg/100 g obtained from the fruit of Califonia wonder. The lycopene contents of these two varieties were significantly better than that of Ogbomoso local (0.41 mg/100 g). Fertilizer types had

Table 3. Effect of fertilizer types on the phosphorus, potassium, calcium and magnesium contents of three variety of tomato.

Fertilizer type	Tomato variety			Mean
	Califonia wonder	Ogbomoso local	Roma VF	
Phosphorus content (g 100 g^{-1})				
0 kg	13.7	22.5	28.5	21.6^{b}
100% NPK	15.6	16.4	16.8	16.3^{e}
100% TC	24.8	17.7	25.2	22.6^{a}
75% NPK + 25% TC	21.7	18.3	18.6	19.5^{c}
50% NPK + 50% TC	15.9	19.3	19.9	18.4^{d}
25% NPK + 75% TC	19.6	26.3	22.4	22.8^{a}
Mean	18.5^{c}	20.1^{b}	21.9^{a}	
Potassium content (g 100 g^{-1})				
0 kg	3.1	5.4	4.2	4.2^{d}
100% NPK	3.6	3.7	5.5	4.2^{d}
100% TC	4.5	4.4	6.1	5.0^{c}
75% NPK + 25% TC	3.9	5.7	7.4	5.7^{b}
50% NPK + 50% TC	4.4	6.3	6.6	5.8^{b}
25% NPK + 75% TC	4.9	9.5	5.9	6.8^{a}
Mean	4.1^{b}	5.8^{a}	5.9^{a}	
Calcium content (g 100 g^{-1})				
0 kg	3.1	5.4	4.2	4.2^{d}
100% NPK	3.6	3.7	5.5	4.2^{d}
100% TC	4.5	4.4	6.1	5.0^{c}
75% NPK + 25% TC	3.9	5.7	7.4	5.7^{b}
50% NPK + 50% TC	4.4	6.3	6.6	5.8^{b}
25% NPK + 75% TC	4.9	9.5	5.9	6.8^{a}
Mean	4.1^{b}	5.8^{a}	5.9^{a}	
Magnesium content (g 100 g^{-1})				
0 kg	15.2	18.6	21.3	18.4^{c}
100% NPK	17.3	19.3	19.5	18.7^{b}
100% TC	18.8	18.6	22.4	19.9^{a}
75% NPK + 25% TC	16.9	17.5	14.6	16.3^{e}
50% NPK + 50% TC	18.3	16.2	17.5	17.3^{d}
25% NPK + 75% TC	14.9	15.7	18.8	16.5^{e}
Mean	16.9^{c}	17.7^{b}	19.0^{a}	

Means followed by the same letter are statistically similar (DMRT, 5%).

significant effect on tomato fruit lycopene content. Application of 100% TC gave the highest value (0.71 mg/100 g), while the least (0.38 mg/100 g) was obtained from non fertilized plants. The interactive effects of the two factors tested showed that Roma VF variety fertilized with 100% TC produced fruits that contained the highest amount of lycopene.

Crop growth, yield and product quality in relation to application of compost or in combination with small doses of mineral fertilizer has been widely reported (Togun et al., 2003). High fruit yield and nutrient contents of plants nourished with organic fertilizer could be due to the fact that the materials not only contained sufficient nutrients but the nutrients are slowly released to the plants. This prevents nutrient loss and leaching, as well as improving nutrient use efficiency. All these facilitate higher production of economic part of the plant. It could be observed that the higher the compost contents of some treatments, the better the plant performance in terms of fruit yield and quality. Many research works have reported higher nutritional values of organically grown vegetables when compared with inorganic ones. This

Table 4. Effect of fertilizer types on some fruit proximate qualities of three varieties of tomato.

Tomato variety	Fertilizer type	pH	Moisture Content (%)	Crude protein (g/100 g)	Crude fibre (g/100 g)	Ether extract (g/100 g)	Total Soluble solid (g/100 g)
Califonia wonder	0 kg	5.38^{b}	90.12^{d}	1.66^{d}	1.33^{c}	0.64^{d}	4.1^{c}
	100% NPK	5.40^{b}	91.78^{c}	1.45^{e}	1.24^{e}	1.03^{b}	4.2^{c}
	100% TC	5.48^{b}	91.89^{c}	2.30^{b}	1.44^{b}	0.93^{c}	6.7^{a}
	75% NPK + 25% TC	5.81^{a}	91.76^{c}	1.73^{d}	1.31^{c}	0.36^{f}	5.6^{ab}
	50% NPK + 50% TC	5.70^{a}	91.64^{c}	1.92^{c}	1.35^{c}	0.77^{c}	6.2^{a}
	25% NPK + 75% TC	5.80^{a}	91.44^{c}	1.80^{d}	1.41^{b}	0.84^{c}	5.9^{a}
	Mean	5.60	91.44	1.81	1.35	0.76	5.45
Ogbomoso local	0 kg	5.02^{d}	90.05^{d}	1.98^{c}	1.72^{a}	1.27^{a}	2.9^{d}
	100% NPK	5.30^{b}	90.08^{d}	2.50^{ab}	1.68^{a}	1.25^{a}	3.9^{c}
	100% TC	5.17^{c}	90.48^{d}	3.08^{a}	1.53^{ab}	1.18^{b}	3.8^{c}
	75% NPK + 25% TC	5.31^{b}	90.66^{d}	2.80^{ab}	1.62^{a}	1.21^{ab}	4.2^{c}
	50% NPK + 50% TC	5.28^{c}	92.68^{b}	1.52^{d}	1.22^{e}	0.41^{e}	5.0^{b}
	25% NPK + 75% TC	5.31^{b}	91.89^{c}	1.40^{e}	1.20^{e}	0.35^{e}	4.1^{c}
	Mean	5.23	90.97	2.21	1.50	0.95	3.98
Roma VF	0 kg	5.38^{b}	92.67^{b}	1.60^{d}	1.28	0.82^{c}	3.9^{c}
	100% NPK	5.40^{b}	92.65^{b}	2.10^{b}	1.34^{c}	0.97^{c}	5.2^{b}
	100% TC	5.50^{b}	93.24^{a}	1.42^{e}	1.29^{d}	0.53^{d}	4.8^{b}
	75% NPK + 25% TC	5.34^{b}	93.45^{a}	1.57^{d}	1.28^{d}	0.45^{d}	5.6^{b}
	50% NPK + 50% TC	5.82^{a}	92.88^{a}	1.48^{d}	1.23^{e}	0.56^{d}	5.2^{b}
	25% NPK + 75% TC	5.80^{a}	94.38^{a}	1.64^{d}	1.38^{c}	0.58^{d}	4.9^{b}
	Mean	5.54	93.21	1.64	1.30	0.65	4.93

Means followed by the same letter along the column are statistically similar (DMRT, 5%).

Table 5. Effect of fertilizer types on fruit vitamin C and lycopene contents of three varieties of tomato.

Fertilizer type	Tomato variety			Mean
	Califonia wonder	Ogbomoso local	Roma VF	
Fruit vitamin C (mg /100 g)				
0 kg	12.4	12.4	16.8	13.9c
100% NPK	22.1	18.9	28.5	23.2b
100% TC	19.6	15.3	28.9	21.3bc
75% NPK + 25% TC	26.7	19.2	38.0	27.9a
50% NPK + 50% TC	26.1	21.6	38.2	28.6a
25% NPK + 75% TC	25.1	15.0	30.4	23.5b
Mean	22b	17.1c	30.1a	
Fruit lycopene content (mg /100 g)				
0 kg	0.41	0.32	0.42	0.38d
100% NPK	0.40	0.31	0.57	0.43c
100% TC	0.78	0.57	0.79	0.71a
75% NPK + 25% TC	0.54	0.42	0.61	0.52b
50% NPK + 50% TC	0.50	0.38	0.63	0.50b
25% NPK + 75% TC	0.49	0.48	0.51	0.48c
Mean	0.52a	0.41b	0.59a	

Means followed by the same letter along the column are statistically similar (DMRT, 5%).

could explain the better quality of tomato fruit nourished with pure compost (100% TC) or that contained high amount of compost. Compost contained many active sites which improved soil cation exchange capacity (CEC) and fertility. This stimulates better nutrient uptake and utilization by the crop. In the present study, combine application of compost with small amount of NPK improved the efficiency of the former.

Conclusion

Application of fertilizer whether organic or inorganic, improved the growth and fruit yield and fruit quality of tomato. Integrated use of organic and inorganic fertilizers was found to be better than using each alone. In this study, combine application of 50% NPK and 50% TC was found to be the best treatment. This same treatment produced the highest fruit yield which compared favourably with what was obtained with 100% NPK treatment.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Physiological and enzymatic changes in rice seeds stored at low temperatures

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This study aimed to evaluate the effect of low temperatures on the physiological and enzymatic changes of rice seeds. The seeds were packed in airtight chambers and maintained at temperatures of 8 and -50°C for periods of 15, 30 , 45, 60, 75 and 90 days. The same procedure was adopted for the control treatment with the seeds kept at a temperature of 25°C. The seeds were evaluated regarding germination test; seedling emergency; emergency speed index; length and dry weight of radicle; and seedling of shoot. The activity of amylase and total protein content were also evaluated. The temperatures of 8 and -50°C significantly influenced the physiological quality and the enzyme amylase activity of rice seeds, resulting in higher germination, seedling emergence and enzyme activity. The temperature is a promising alternative for the maintenance of physiological quality and enzymatic activity of rice seeds during storage.

Key words: *Oryza sativa* L., enzymatic activity, physiological quality, storage.

INTRODUCTION

The conservation of seed quality during the storage period is a key factor to be considered in the complex system of seed production, since the establishment in the field is directly related to the physical, physiological and sanitary conditions of the seed. Among the factors that determine the maintainability of the quality of seeds during storage are natural or modified conditions that favor their conservation (Carvalho and Villela, 2006), such as the storage temperature (Caldwell et al., 2005; Toledo et al., 2009). Temperature plays a main role in influencing the rates of biochemical processes and indirectly affecting the water content of the seeds. Therefore, the period of seed viability can be increased by reducing the humidity and storage temperature. This reduces the respiratory rate of the seeds and, therefore, minimizes the degradation of its reserve tissue (Bewley

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Abbreviations: ESI, Emergence speed index; **PVP,** polyvinylpyrrolidone.

and Black, 1994; Takaki, 2004). Low storage temperatures favor the maintenance of biochemical processes in the embryo, subsequently allowing normal seedling development and uniform germination. High temperatures, according to MacDonald (2000), may denature proteins and alter membrane permeability, thereby, accelerating deterioration.

The initial maintenance of physiological processes of seed depends on structural enzymes which have specific requirements for temperature (Bewley and Black, 1994). During seed germination, the accumulated material in starchy endosperm is mobilized by enzymes that are synthesized and secreted (Devi et al., 2007). Among the enzymes, the amylases are predominantly synthesized during germination and they hydrolyze the starch granules to produce monosaccharides, which are energy source for the development of seedlings (Helland et al., 2002). Generally, the α-amylase hydrolyzes the starch granules; β-amylase and α-glucosidase have a minor role only in the hydrolysis of dextrin, an α-amylase product. In this sense, for the quality control program of seed production, monitoring the amylase activity in rice seeds is extremely important, especially when the objective is to produce viable and of high quality seeds. Thus, the test of α-amylase may be of fundamental importance to evaluate the physiological potential of seeds after storage period (Panobianco et al., 2007).

This study aimed to evaluate the physiological changes that occur in rice seeds stored at low temperatures.

MATERIALS AND METHODS

The cultivars of the rice seeds used were IRGA 423 and 424, obtained from seed producers in the municipalities of Formoso do Araguaia (11°47'48" S latitude, 49°31'44 "W longitude) and Lagoa da Confusão (10°47'37" S latitude, 49°37 '25" W longitude), at the state of Tocantins - Brazil. After the standardization of the humidity at 11%, the seeds were placed in airtight chambers with volume of 2 L and stored at the temperatures of 8 ± 1°C and -50 ± 1°C, the control kept in paper bags at room temperature around 25°C. The seeds remained stored during periods of 15, 30, 45, 60, 75 and 90 days.

Germination test

Initially, the dormancy and initial germination was determined with the seeds that were obtained after the moisture uniformity. Four samples of 50 seeds, for each repetition, were placed on germitest paper moistened with sterile distilled water at a ratio of 2.5 times the weight of paper and placed in a climatic chamber at 25°C. At 14 days after seeding, there was the final count of germination, by determining the percentage of normal seedlings (Brasil, 2009).

Length and dry weight of seedlings

The evaluation was performed according to the method described by Vanzolini et al. (2007), with seedlings that originated during the germination test, randomly constituting four replications of 10 seedlings. The shoot and root length (cm.plântula^{-1}) was determined through a millimeter ruler, with the mean obtained by the sum of each repetition divided by the number of normal seedlings. To determine the dry mass, four replications of ten normal seedlings were placed in paper bags and taken to the greenhouse at 70°C until constant weight. The weigh-in was held in 0,001 g precision scale and the data were expressed in grams seedling^{-1}.

Seedling emergence

It was performed according to the method used by Fleck et al. (2003) using plastic trays with dimensions of 50 × 25 × 15 cm; the seeds were sown in 10 cm thick at properly sterilized sand. At the end of each storage period (15, 30, 45, 60, 75 and 90 days), four replications of 100 seeds of each cultivar were performed. The emerged seedlings were performed at five periods (6, 8, 10, 12 and 14 DAE) and the results expressed as a percentage of normal seedlings. For the emergence speed index (ESI), we used the formula Maguire (1962), in which ESI = N1 / N2 + D1 / D2 + ... + Nn / Dn where: ESI = seedling emergence speed index; N = number of emerged seedlings, computed from first to last count; D = number of days from sowing of first to last count.

Specific activity of amylase

The evaluation of the specific activity of amylase was performed with seeds after 90 days storage. The germination of seeds was induced by incubating them in Petri dishes lined with filter paper moistened with distilled water and kept at room temperature. After induction of germination at periods of 0, 12, 24, 48, 72 and 96 h, the amylase extraction was performed according to the methodology used by Jose et al. (2004). The extractions were performed through maceramento - seeds in the presence of liquid nitrogen (N_2) in porcelain mortar. For each treatment, 4 extractions were performed, each extraction constituted in a repetition. Then, 200 mg of seed powder were suspended in 600 µL of extraction buffer (Tris – HCl 0.2 M (pH = 8.0) with 0.4% of PVP (Polyvinylpyrrolidone) where they remained under agitation in table at 6°C for 12 h. After this period, the solution was transferred to Eppendorf tubes and centrifuged at 4000 rpm for 1 h at 4°C. After, a process for achievement of the enzyme extract was held in order to review the specific amylase activity using the methodology described by Miller (1959), which were used 40 µL for each treatment of the extracts in 60 µL of 50 mM sodium acetate buffer, pH = 5.5 with 100 uL of 0.5% starch solution and maintained for 3 h of incubation at 50°C. After that, the reaction was quenched by the addition of 1000 µL DNS reagent and boiled again for 10 min. Reducing sugars present in the enzyme extract were deducted from the calculation of enzyme activity. For this, 40 µL of the enzyme extract were added to 160 µL of the same buffer and the mixture was incubated under the same test conditions prior to the addition of reagent DNS. Reducing sugars were determined by the DNS method compared to a calibration curve prepared with glucose solution at different concentrations within the same test conditions. The absorbance was read with a spectrophotometer at a wavelength of 540 ηm zeroed with white consisting of 40 uL of distilled water, 160 µL of the same buffer and 1 mL of DNS. Reading for dilutions were performed when necessary. The activity was expressed as µmol of reducing sugar released per minute of reaction per mg of total protein.

Determination of total proteins

The determination of the total protein present in the extracts of the seeds was carried out according to the methodology described by

Table 1. Percentage of germination (G), seedling emergence (SE) and the emergence speed index (ESI) in seeds of rice (cultivars IRGA 423 and 424) depending on room temperature (25, 8 and -50°C); and periods of storage (15, 30, 45, 60, 75 and 90 days).

Cultivar		Storage (days)																	
		15			30			45			60			75			90		
		Temperature (°C)																	
		25	8	-50	25	8	-50	25	8	-50	25	8	-50	25	8	-50	25	8	-50
Irga423	G	83^{a}	92.5^{a}	91^{a}	82.7^{a}	93.5a	90^{a}	83^{a}	94^{a}	93^{a}	79.2^{b}	93.2^{a}	92.5^{a}	76.7^{b}	93^{a}	91.7^{a}	68.7^{b}	93.7^{a}	90^{a}
	SE	83.6^{a}	92^{a}	92.3^{a}	83.3^{a}	94^{a}	93^{a}	78.6^{b}	93^{a}	92^{a}	76^{b}	94^{a}	94.2^{a}	73^{b}	91^{a}	92.2^{a}	66.6^{b}	91^{a}	93^{a}
	ESI	51.5^{b}	64.2^{a}	64.7^{a}	50.8^{b}	62.9^{a}	64.6^{a}	54.4^{b}	71.7^{a}	69.6^{a}	52.9^{b}	62^{a}	61a	60,6^{b}	66.7^{a}	67.7^{a}	54.2^{b}	71.3^{a}	71.1^{a}
	VC(%)	9.0	7.8	10.2	9.3	8.7	8.0	7.4	12.4	10.6	6.4	9.7	8.4	9.7	13.8	14.9	9.4	11.8	13.9
Irga424	G	91.2^{a}	91.7^{a}	92.2^{a}	90.5^{a}	92^{a}	93.2^{a}	90.5^{a}	92.5^{a}	92^{a}	83.5^{a}	93^{a}	92.7^{a}	83.5^{b}	92.7^{a}	92.5^{a}	80.5^{ab}	93^{a}	91.2^{a}
	SE	94.3^{a}	92^{a}	93^{a}	91^{a}	92^{a}	92.3^{a}	90.3^{a}	92.3^{a}	92.3^{a}	83^{a}	93^{a}	92.3^{a}	82^{a}	92.3^{a}	92.6^{a}	81^{b}	93^{a}	92.8^{a}
	ESI	57.8^{a}	63.2^{a}	71.1^{a}	63^{a}	70.9^{a}	62.6^{a}	62.3^{a}	71.2^{a}	75.2^{a}	58^{b}	69^{a}	61.8^{a}	61.5^{a}	68.2^{a}	72.1^{a}	60.5^{ab}	73.1^{a}	76.2^{a}
	VC(%)	11.8	8.9	8.7	9.0	14.8	12.3	10.9	8.7	6.9	7.9	9.8	8.6	11.9	15.9	7.9	8.0	7.2	12.9

Means followed by the same lower case letter in the line do not differ by Tukey test (P ≤ 0.05); CV: Coefficient of variation.

Bradford (1976), being used at a rate of 100 uL of sample extract with 1000 uL of Bradford reagent (Kit BIORAD®), with four replications per treatment. The quantitation of total protein was calculated through comparison with a calibration curve prepared with different concentrations of bovine serum albumin under the same test conditions. Readings were taken in a spectrophotometer at a wavelength of 595 ηm and the values expressed in mg/mL.

Amylase activity gel SDS-PAGE

The polyacrylamide gel was made 4.5% (concentrating gel) and 7.5% (separating gel containing 5% of soluble starch). The analysis of amylase activity was performed through the use of electrophoresis system under non-denaturing conditions as described by Jose et al. (2004) using a vertical electrophoresis Loccus Biotecnologia® as apparatus. The samples were suspended another time in 1X sample buffer (50 µL of Tris-HCl 0.5 mmol L^{-1} buffer pH 6.8, 100 µL glycerol, 0.005 mg of bromophenol blue and enough distilled water for 1.0 mL), boiled for 5 min in water bath and was applied in the gel. The race was made using a voltage of 100 V and 80 mA. After the run, the gels were stained by placing them on resublimed iodine for visualization of starch degradation bands.

Experimental design

The experimental design was completely randomized in a factorial arrangement 6 × 3 × 2, consisting of storage periods (15, 30, 45, 60, 75 and 90 days), three temperatures (room temperature: 25, -50 and 8°C) and two cultivars (IRGA 423 and 424). The comparison of means was performed through Tukey test (P≤0.05) using the statistical program SISVAR 5.0 (Ferreira, 2003).

RESULTS AND DISCUSSION

Germination

The control seeds were kept at a temperature of 25°C (Table 1). Temperatures above 25°C are considered harmful to maintain the physiological quality of rice seeds, depending on the storage period, due to the physiological and biochemical changes that occur gradually (Marini et al., 2012). Germination of IRGA 423 was lower when stored at room temperature (average 25°C) after 15 days storage (Table 1). For IRGA 424, the percentage of germination was affected negatively and significantly by the temperature after 70 days of storage, at 25°C. Storage temperature directly influences the activity of gas exchange in seeds. Mild temperatures decrease the rate of gas exchange (Patane et al., 2006), favoring the maintenance of physiological quality of stored seeds, verified by the highest percentage of germination. The results of the emergency speed index were similar to the results of the germination test (Table 1). For both varieties stored at 8°C and -50°C, significant differences became most pronounced in 90 days in relation to seeds stored at room temperature, resulting in a reduction of the emergence speed. Reducing the emergency speed of seeds stored at room temperature may be related to the consequent deterioration, which is probably associated to temperature fluctuations and varying humidity, occurring on site during storage. Tunes (2014) showed that seeds stored under conditions of temperature fluctuation and

Table 2. Length and dry weight of shoot radicle and rice seedlings (cv. IRGA 423) depending on room temperature (25 ° C), -50 ° C and 8°C, storage periods (15, 30, 45, 60, 75 and 90 days).

Periods (days)	Radicle length (cm)			Aerial part length (cm)		
	25°C	08°C	-50°C	25°C	08°C	-50°C
15	12.8[a]	13.4[a]	13.2[a]	16.1[a]	16.0[a]	15.9[a]
30	12.0[a]	12.8[a]	13.0[a]	15.5[a]	16.1[a]	16.0[a]
45	11.8[a]	12.5[a]	13.1[a]	13.6[b]	15.9[a]	16.2[a]
60	10.7[b]	11.7[a]	12.9[a]	12.4[b]	16.5[a]	16.1[a]
75	8.1[c]	11.2[b]	13.5[a]	12.3[b]	16.8[a]	16.5[a]
90	6.5[c]	11.5[b]	13.3[a]	9.8[b]	15.6[a]	15.8[a]
VC%	8.31			8.16		
Periods (days)	**Weight of radicle (g)**			**Weight of Aerial Part (g)**		
	25°C	08°C	-50°C	25°C	08°C	- 50°C
15	0.0126[a]	0.0128[a]	0.0131[a]	0.0180[a]	0.0181[a]	0.0182[a]
30	0.0127[a]	0.0132[a]	0.0132[a]	0.0172[ab]	0.0184[a]	0.0184[a]
45	0.0125[b]	0.0133[ab]	0.0137[a]	0.0175[a]	0.0176[a]	0.0180[a]
60	0.0107[a]	0.0129[a]	0.0129[a]	0.0145[a]	0.0172[a]	0.0178[a]
75	0.0090[b]	0.0131[a]	0.0132[a]	0.0131[b]	0.0171[a]	0.0176[a]
90	0.0091[b]	0.0133[a]	0.0130[a]	0.0127[b]	0.0170[a]	0.0172[a]
VC%	6.11			6.42		

Means followed by the same lower case letter in the line do not differ by Tukey test (P≤0.05).

varying humidity may have reduced viability compared with those stored under constant temperature and humidity.

In general, the results obtained also show that the storage temperature is an important factor for the preservation of rice seeds, directly influencing the physiological seed quality. Storage at low temperatures (-50 and 8°C) promoted the conservation of seeds of both cultivars (IRGA 423 and 424). This process may be associated to the decrease of respiration and, consequently, reduction of the metabolism and maintenance of enzymes, reserve tissues and membrane permeability, as evidenced by higher germination during storage up to 90 days compared to the control (Table 1). According to Filho (2005), low temperature conditions allow for the reduction of seed metabolism, contributing to a longer life, while higher temperature conditions produce a more rapid loss of seed viability and the accelerated metabolic reactions often result in increased water content.

Length and dry weight of seedling

The results for radicle (Fc = 31.750; Pr > Fc = 0.004) (P ≤ 0.05) and for shoot (Fc = 42.313; Pr > Fc = 0.001) (P ≤ 0.05) of seedling for both cultivars (IRGA 423 and 424) showed superiority of seedlings originated from seeds stored at low temperatures (Table 2). Seeds that were stored at 25°C originate seedlings with shorter length of the primary root and shoot, after 60 days of storage for IRGA 423 and 30 days for IRGA 424 (Tables 2 and 3). Seedlings originated from seeds stored at -50 and 8°C that remained with similar sizes from beginning until the end of the experiments, especially the ones -50°C, which showed higher values than at the end of 90 days of storage (Tables 2 and 3).

With the dry weight of shoot and radicle, it was observed that the seeds of both cultivars (IRGA 424 and 423), kept at room temperature conditions, had their dry matter reduced, especially for IRGA 424, with a significant difference at 90 days storage; while at low temperature treatment (-50 and 8°C), maintained constant mass at storage periods (Tables 2 and 3). This may be due to the inhibition of respiration and, thus, the preservation of the reserves of seeds (Zuchi and Bevilaqua, 2012).

Specific amylase activity

The specific activities of amylase were more significant for cultivar IRGA 423 than for cultivar IRGA 424 among all established treatments (Figure 1A and 1B). Seeds stored at room temperature did not show a significant increase in amylase activity (Figure 1A). However, the seeds of IRGA 423 stored under refrigeration at 8 and -50°C for 90 days showed a significant increase in amylase activity in the first 12 h of germination, reaching approximately 0.5 up to 0.8 IU / mg, respectively. There was a decline of activity within 24 h up to 96 h and consequent increase in germination. The same effect was observed in the specific activity of amylase for seed

Table 3. Dry mass and length of the primary root and shoot of rice seedlings (cv. IRGA 424) depending on room temperature (25, 8 and -50°C) and storage periods (15, 30, 45, 60, 75 and 90 days).

Período	Radicle length (cm)			Aerial part length (cm)		
(days)	25°C	08°C	-50°C	25°C	08°C	-50°C
15	13.7[b]	13.7[b]	13.2[ab].	16.5[a]	16.0ab	16.5[a]
30	12.2[b]	13.5[a]	13.6[a]	13.9[c]	16.6a	16.3[ab]
45	11.3[b]	13.4[a]	13.5[a]	13.1[b]	15.8ab	16.5[a]
60	11.1[c]	13.7[ab]	13.3[a]	13.3[b]	15.5a	15.9[a]
75	8.3[c]	12.9[ab]	13.6[a]	12.6[b]	15.3a	16.0[a]
90	6.9[b]	13.0[a]	13.2[a]	10.2[b]	14.9ab	15.7[a]
CV%		9.16			7.43	

Período	Weight of radicle (g)			Weight of Aerial Part (g)		
(days)	25°C	08°C	-50°C	25°C	08°C	- 50°C
15	0.0140[ab]	0.0135[a]	0.0136[a]	0.0180[a]	0.0185[a]	0.0187[a]
30	0.0130[b]	0.0137[ab]	0.0135[a]	0.0170[a]	0.0180[a]	0.0188[a]
45	0.0111[a]	0.0131[a]	0.0133[a]	0.0172[a]	0.0183[a]	0.0186[a]
60	0.0112[a]	0.0130[a]	0.0136[a]	0.0161[a]	0.0179[a]	0.0188[ab]
75	0.0104[a]	0.0129[a]	0.0133[a]	0.0158[a]	0.0174[a]	0.0184[a]
90	0.0097[c]	0.0126[b]	0.0131[ab]	0.0128[b]	0.0176[a]	0.0183[a]
CV%		7.51			7.13	

Means followed by the same lower case letter in the line do not differ by Tukey test (P≤0.05).

IRGA 424 stored at low temperatures for 24 up to 48 h of germination (Figure 1B). The amylase activity tends to increase significantly as the germination process is intensified, which may also be linked to the fact that low temperatures induce the biosynthesis of gibberellin - the precursor of biosynthesis of the amylase hormone (José et al., 2004; Neves and Moraes, 2005). Veluppillai et al. (2009) observed, from the first day, a significant increase in rice seed germination through the reduction of sugars and endogenous amylase activity; followed by a linear sharp rise on the third day of germination. Amylase expression during grain germination of cultivation can vary from one to another (Nandi et al., 1995), which was also observed in this study. Excluding only the amylase expression as an important factor, it is possible to affirm that the practice of seed germination has been used to improve their nutritional value. Germination has an important effect on the chemical composition, nutritional value and acceptability of the product for human consumption (Bailly, 2004).

Amylase activity gel SDS-PAGE

Through the analysis of the amylase activity in non-denaturing SDS-PAGE containing 5% starch, it is clearly observed that amylase activity was seen in the starch and it was possible to see starch degradation bands in the gels (Figure 2). Regarding both cultivars, it is possible to see that the amylase activity was more pronounced during germination, and that low temperature (-50 and 8°C) treatments stand out in comparison to the control treatment, especially within 96 hours of germination. These results corroborate with those found in the specific activity of amylase (Figure 1), in which the seeds stored under low temperatures (-50 and 8°C), for 90 days, presented higher specific activity of amylase during germination, in comparison to the control.

Total protein

The statistical analysis of total protein content recovered from all treatments during the germination period showed no significant differences by Tukey test at 95% confidence (Figure 3A and 3B). The total protein content and constant during the process of germination of rice seeds for 5 days, was also observed by Veluppillai et al. (2009). According to these results and considering that the specific activity is the amount of amylase activity per mg protein (UI/mg), it is possible to infer that the amylase expression occurred with greater intensity during germination of seeds that were stored at low temperatures (Figures 1A and 1B). For Petruzzelli and Taranto (2010) the development of amylase activity is an important event, which can be detected at the beginning of seed germination, and its primary role is to provide substrates for seedling use until they become photosynthetically independent. Therefore, the high specific activities of amylase observed in treatments performed with seeds stored at low temperatures for 90 days confirm the results of germination, seedling emergence

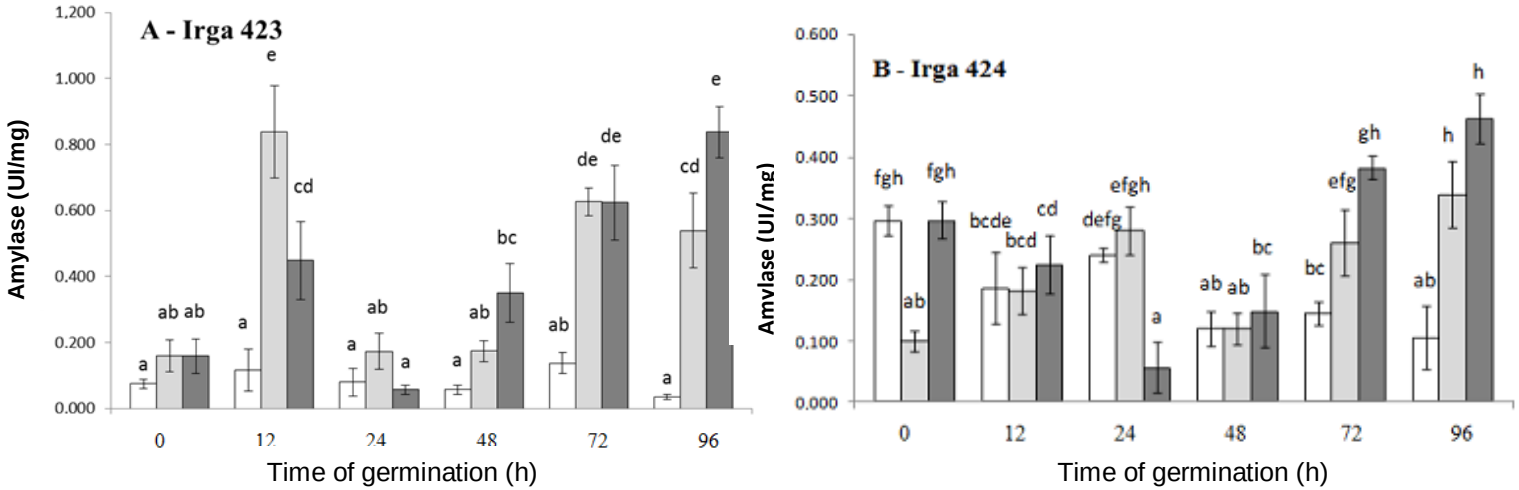

Figure 1. Activity of amylase extracted from rice seeds (*Oryza sativa* L.) after 90 days of storage under treatments. ☐ room temperature; ▒ 8°C and ■ -50°C. **A =** Cultivar IRGA 423 and **B =** Cultivar IRGA 424. Means followed by the same lower case letter in the columns do not differ by Tukey test at 95% confidence. The bars represent the standard error.

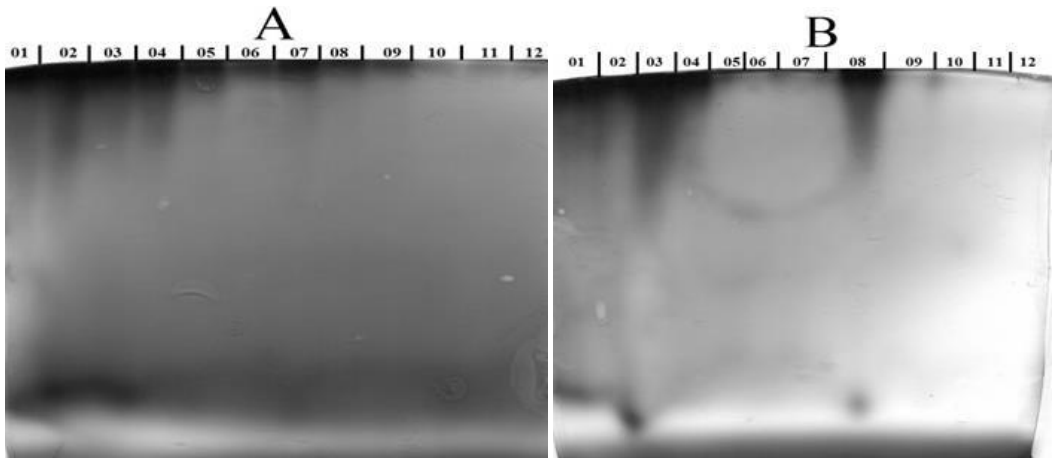

Figure 2. Evaluation of amylase activity extracted from rice seeds (*Oryza sativa* L.) polyacrylamide gel (5% starch) after 90 days of storage at room temperature (25°C), -50 and 8°C. **A**- Cultivar IRGA 423 and **B-** Cultivar IRGA 424. Sequence: **1**) control: 12 h of germination; **2**) 08°C: 12 h of germination; **3**) -50°C: 12 h of germination; **4)** control: 48 h of germination; **5)** 8°C: 48 h germination; **6**) -50°C: 48 h germination; **7**) control: 72 h of germination; **8**) 08°C: 72 h germination; **9)** -50°C: 72 h germination; **10**) control: 96 h of germination; **11**) 08°C: 96 h of germination; and **12**) -50°C: 96 h of germination.

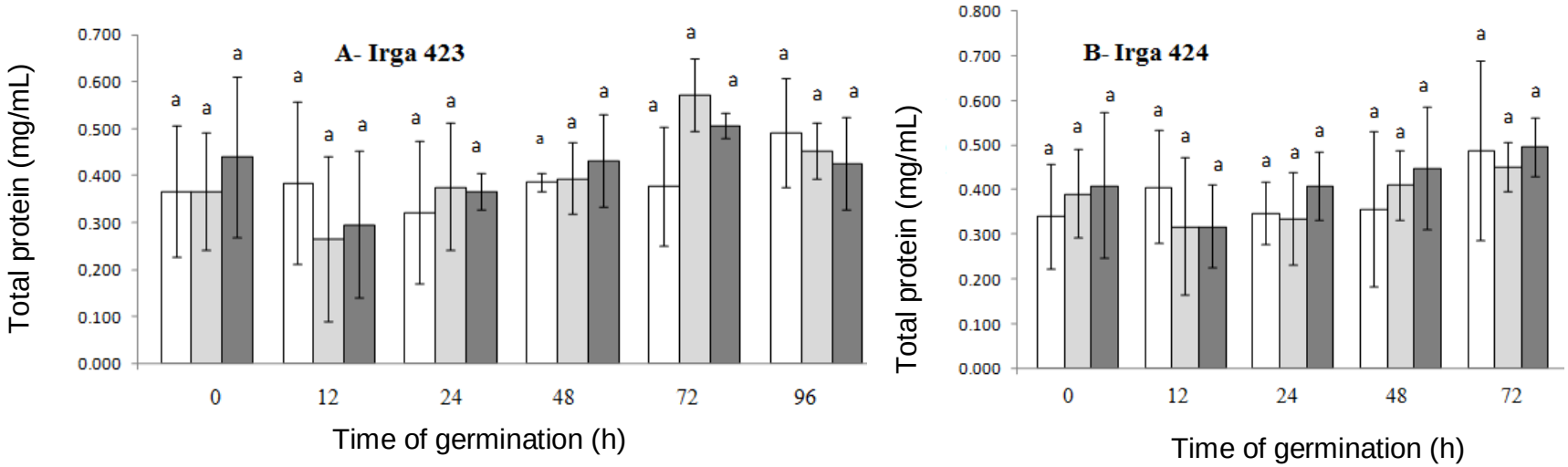

Figure 3. Total protein extracted of rice seeds (*Oryza sativa* L.), during germination after 90 days of storage under treatments: ☐ room temperature ▨ 8°C and ■ -50°C. The bars represent the standard error.

and emergence speed index of rice seeds (Table 1).

According to José et al. (2004), when seeds are exposed to high temperatures, the cellular membrane system ruptures and disintegrates, possibly due to changes in the constituent lipids. The solubility and protein binding capacity can also be reduced, causing injuries to the mitochondrial structure, thereby affecting the respiratory rate, and other subcellular systems. This results in metabolic and biochemical changes that are involved in the prevention of injuries caused by high temperatures, which directly interferes with amylase activity during germination.

Based on germination, emergence speed and seedling quality assessments, the reserve tissues of seeds stored under uncontrolled conditions may have been affected by the deterioration caused by high temperatures during storage. The starch in the seeds' reserves and, consequently, the expression of amylase activity may also have been affected by this deterioration. Spinola et al., (2000), concluded that monitoring enzyme change, rather than physiological assessment markers, may be more effective in detecting metabolic alterations indicating the beginning of the seed decay process during storage. However, the cultivars IRGA 423 and 424 stored at low temperatures remained protected from these deleterious effects, which allowed them to express their genetic potential.

In general, storage temperature influenced the physiological quality of rice seeds. Although inevitable and irreversible, the process of seed deterioration can be reduced by storage at appropriate temperatures. At low temperatures, the biochemical and physiological changes that cause seed deterioration are reduced, resulting in the maintenance of seed quality and, therefore, the quality of seedlings. Seeds stored at temperatures of −50°C and 8°C had higher germination rates compared with seeds stored at 25°C. Temperature is an important factor for the conservation of seeds, directly affecting the speed of biochemical processes and interfering with the increased amylase enzyme activity, making the activity favorable during the lag in rice seed germination.

Conflict of interests

The authors did not declare any conflict of interest.

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The mineral composition of five insects as sold for human consumption in Southern Africa

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Edible insects have been proposed as an alternative protein source that is economically and environmentally preferable to livestock, and certain species may be high in nutrients that benefit human health. We present data describing the mineral content of five edible insects as sold in South Africa and Zimbabwe. We report high variation between and within species, and note that these insects contain significant quantities of potentially beneficial, and potentially harmful, micronutrients. Two caterpillars were notably high in Fe and Zn, which are important nutrients for combating iron deficiency anemia. Na content varied both between and within species, suggesting that some sellers add quantities of salt that could be harmful to health. Mn levels were high in edible termites. We concluded that caterpillars can be promoted as nutrient rich foods in southern Africa; that added salt should be limited in commercial products; and that further research is required to determine whether common serving sizes of termites may put consumers in danger of manganese poisoning.

Key words: Edible insects, nutrition, mineral composition, micronutrients, Lepidoptera.

INTRODUCTION

The global food system is approaching crisis. Land clearance for agriculture is causing accelerated environmental degradation (Green et al., 2005), yet demand for food continues to increase as the world population continues to rise (Godfray et al., 2010). The intensive production and promotion of insects as human food and animal feed has been suggested as one strategy to combat this situation, most notably in two landmark reports published in 2010 and 2013 by the Food and Agriculture Organisation of the United Nations (FAO) (Durst et al., 2013). When farmed, insects have a significantly lower environmental impact compared with traditional livestock, as they use less land and energy (Oonincx and De Boer, 2012), have a higher feed

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Abbreviations: DALYs, Disability-adjusted life years; **Na,** sodium; **K,** potassium; **Ca,** calcium; **Mg,** magnesium; **Al,** aluminium; **P,** phosphorus; **S,** sulphur; **Cu,** copper; **Fe,** iron; **Mn,** manganese; **Zn,** zinc; **DRI,** daily recommended intake.

conversion ratio (DeFoliart, 1995), and produce fewer carbon emissions (Oonincx et al., 2010; Vantomme et al., 2014). Based on this evidence, edible insects are one potential 'panacea' to the crisis facing the world food system. Currently, malnutrition is the most important risk factor for illness and death on a global scale, and women are disproportionately affected (Müller and Krawinkel, 2005). Malnutrition comprises not only protein-energy malnutrition but also micronutrient deficiencies, which can lead to death and disease as well as impairment in mental and physical development (Olness, 2003; Rivera et al., 2003). Recently, insects have been advocated as nutritionally preferable to other protein sources (Van Huis et al., 2013). Previous studies have shown that several edible insects are high in certain micronutrients (Bukkens, 1997; Belluco et al., 2013), and therefore they may have potential as an important food in combating problems of under-nutrition and micronutrient deficiency. Specifically, many edible insects are high in iron and zinc (Kinyuru et al., 2012). Both of these nutrients are crucial in combating iron deficiency anemia, which is the most prevalent nutritional disorder worldwide and impairs both physical and cognitive ability, thus reducing the work productivity of adults and the learning capacity of children (Armstrong and Summerlee, 2014). Iron and zinc deficiencies combined are responsible for 2.4 and 1.9% of disability-adjusted life years (DALYs) respectively (Black, 2003). Of the insects sold in southern Africa the mopane caterpillar (*Imbrasia belina*) in particular has been highlighted as containing high quantities of these nutrients (Van Huis et al., 2013).

One common way of addressing dietary deficiencies in essential minerals is through supplementation programmes, but compliance is often low, reducing the efficacy of such schemes (Schultink, 1996). If commercially available edible insects are indeed high in key micronutrients it may be preferable to redirect investment to promoting increased consumption of these traditional foods, which are culturally acceptable foods that are already part of the diet. This would have the added advantage of reducing dependency on food supplementation programmes and imported products.

In order to contribute to the evidence regarding the potential of edible insects to address the public health challenge of micronutrient deficiency in this region, we present data on the mineral content of commercially available insects as sold in multiple locations in Zimbabwe and one from South Africa. The insect species analysed in our study are nutritionally and economically significant across Zimbabwe and South Africa: approximately 318 and 133 tonnes, respectively, of Lepidopteran and Isopteran species are harvested and sold as food in Zimbabwe annually (Dube et al., 2013), and the trade in *I. belina* in South Africa alone is estimated to be worth US$30-50 million per annum (Makhado et al., 2014). *I. belina* and Macrotermes spp are consumed by over 50% of the population of Zimbabwe, including approximately 60% of 16 to 25 year olds and up to 95% of people aged over 55 (Dube et al., 2013).

MATERIALS AND METHODS

We collected samples of commercially available insects in Zimbabwe and in the Limpopo province of South Africa by purchasing insects from markets during 2013 and 2014. Details of specific locations are shown in Table 1. Two samples were received directly during village (Majuru; soldier caste *Macrotermes* spp) and farm (Madora; *I. belina*) visits during the same period. Insects sold at urban markets have undergone treatment to ensure that they are stable at room temperature, using one or a combination of drying, salting and roasting methods. Sellers did not give consistent or detailed descriptions of these processing steps, because no sellers had collected or processed the insects themselves. Therefore, samples were placed 'as sold' in plastic containers to avoid contamination, stored at room temperature and transported to Japan. Mineral content analyses were conducted in the Laboratory of Forest Environment and Resources, Nagoya University, Japan. Three (3) individual caterpillars were homogenized for each caterpillar sample, two *Encosternum delorgugei* were homogenised for each *Encosternum* sample, approx. 4 g of alates were homogenised for each termite alate sample, and 2.5 g of termite soldiers were homogenized for the termite soldier (Majuru) sample. Two replicate analyses were performed for each sample, and the results presented here represent the average of the two replications. Since dried caterpillars are usually washed prior to cooking in Zimbabwe, samples of purchased caterpillars were also washed, using ultrapure water. *Macrotermes* and *Encosternum* are sold in a ready-to-eat state, and therefore these samples were not subject to this treatment.

All samples were freeze dried (FDU-1200 EYELA, TOKYO RIKAKIKAI Co., Ltd., Tokyo, Japan). Before and after drying, we weighed the samples to determine the water content. Samples were then homogenized using a mill mixer with titanium-coated stainless blades (BL-229, SUN Co., Ltd., Osaka, Japan), and subject to wet digestion under reflux with nitric acid and hydrogen peroxide (Matusiewicz, 2003) using a high purity PFA Teflon vessel. After filtering the solutions with a 0.45 µm mixed cellulose ester membrane filter (A045A025A, ADVANTEC, Tokyo, Japan), we determined sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), aluminium (Al), phosphorus (P), sulphur (S), copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) concentrations by inductively coupled plasma atomic emission spectrometry (ICP-AES; IRIS ICARP, Jarrell Ash Nippon Corp., Japan). This is an accepted analytical method for determining mineral composition of food samples, and is the preferred method for Zn, Fe and Cu (Greenfield and Southgate, 2003). For the analysis process, we used ICP standard solutions (Standard A (Al, Bi, Ni, Pb each 100 mg/L in 1 mol/L HNO_3), Standard B (B, Cd, Cr, Zn each 100 mg/L in 0.1 mol/L HNO_3), Standard C (Co, Cu, Fe, Mn each 100 mg/L in 0.1 mol/L HNO_3), and Standard D (Ba, Ca, K, Mg, Na, Sr each 100 mg/L in 0.1 mol/L HNO_3)) purchased from Kanto Chemical Co., Inc., Japan. Standard solutions were diluted to a desired concentration with 0.1 mol/L HNO_3 and ultra-pure water. To evaluate this analysis method, we checked the precision of ICP-AES by analyzing reference sample (NIES CRM No.1 Pepperbush; NIES CRM No. 7 Tea Leaves; NIES CRM No.9 Sargasso, Environment Agency National Institute for Environmental Studies Tsukuba, Ibaraki, Japan). The certified values or reference values of those materials is determined for elements including Na, K, Ca, Mg, Al, P, S, Cu, Fe, Mn, Zn. Detection limit value (ppb) of each elements were Na (3), K (24.3), Ca (627), Mg (5.7), Al (80.7), P (7.2), S (51.6), Cu (27) Fe (5.1), Mn (0.6), Zn (0.3).

Table 1. Details of samples used for mineral composition analysis.

Sample ID	Local name	Species	Preparation[1]	Source	Country	City/Town/Village
1	Madora	*Imbrasia belina*	Washed	Supermarket	Zimbabwe	Rusape
2		*Imbrasia belina*	Washed	Farm	Zimbabwe	Gwanda
3		*Imbrasia belina*	Washed	Market	Zimbabwe	Bulawayo
4		*Imbrasia belina*	Washed	Market	Zimbabwe	Bulawayo
5	Gandari	*Gynanisa maia*	Washed	Market	Zimbabwe	Bulawayo
6		*Gynanisa maia*	Washed	Market	South Africa	Tzaneen
7		*Gynanisa maia*	Washed	Market	Zimbabwe	Mutare
8	Ishwa	*Macrotermes spp (alate)*	As sold	Market	Zimbabwe	Mutare
9		*Macrotermes spp (alate)*	As sold	Market	Zimbabwe	Mutare
10		*Macrotermes spp (alate)*	As sold	Market	Zimbabwe	Nyika
11	Majuru	*Macrotermes spp (soldier)*	Washed	Village	Zimbabwe	Djairo
12	Amanondu	*Cirina forda*	Washed	Market	Zimbabwe	Bulawayo
13		*Cirina forda*	Washed	Market	Zimbabwe	Bulawayo
14		*Cirina forda*	Washed	Market	Zimbabwe	Bulawayo
15	Haruwa	*Encosternum delegorguei*	As sold	Market	Zimbabwe	Masvingo province
16		*Encosternum delegorguei*	As sold	Market	Zimbabwe	Masvingo province
17		*Encosternum delegorguei*	As sold	Market	Zimbabwe	Masvingo province

Caterpillars were washed before analysis, as this is the common practice in Zimbabwean households. *Macrotermes* and *Encosternum,* however, are not usually washed after purchase, as they are sold in a ready-prepared (boiled/fried and salted) state. These insects, therefore, were not washed before analysis.

RESULTS AND DISCUSSION

Table 1 shows details of the samples used for mineral composition analysis. We analysed a total of 17 samples, representing five species of edible insects. All samples were purchased from markets with the exception of two *I. belina* samples, which were purchased at a farm and a supermarket, respectively, and one sample of *Macrotermes* soldiers. The *Macrotermes* soldiers were obtained at a village in Northeastern Zimbabwe, and were freshly collected for use as food. Table 2 shows the mineral content of each insect sample per 100 gas sold, and the Daily Recommended Intake (DRI) value for each mineral where available. Mineral content varies both between and within species. When compared with DRI values, Fe and Zn content are notably high in all samples, with 100 g providing over and above the DRI of Fe in 11 of 17 samples, and of Zn in only 1 sample (*E. delegorguei*). However, the quantity of these and other minerals that are essential for human health are not consistent within species. For example, Ca ranges from 203 to 810 mg in *I. belina*, and 112 to 564 mg in *Gynanisa maia.* Similarly, Zn ranges from 12 to 36 mg in *I. belina*, and Fe ranges from 9 to 57 mg in *G. maia*. These inconsistencies indicate that the mineral content of wild-harvested insects is likely to be influenced by the soil composition and/or diet at their original location, although the nature of this relationship clearly requires further research.

Na content also varies greatly within species, ranging from 13 to 1501 mg in *I. belina* samples, and from 12 to 2674 mg in *G. maia* samples. This variation is likely to be due to salt that is added to insects during processing by some vendors. As a result, the amount of Na in commercially available insects ranges from negligible to amounts that exceed DRI values. This is supported by comparing results from previous studies; for example reported of Na content per 100 g in *Cirina forda* varies from 44.4 mg (Osasona and Olaofe, 2010) to 210 mg (Akinnawo and Ketiku, 2000), and in *I. belina* from 33.3 mg (Kwiri et al., 2015) to 1032 mg (Rumpold and Schluter, 2013). The variation within species, particularly for key micronutrients, is also evident when our values are compared with previously published data. *I. belina* has been reported to have an average Fe content of 12.7 mg (Rumpold and Schluter, 2013) and 31 mg (Kwiri et al., 2015) per 100 g of dry weight in previous studies. These are already disparate values, and far lower than our range of 63 to 130 mg for fresh weight as sold. In the same species, Zn content is similarly varied according to prior estimates, at 14 mg (Rumpold and Schluter, 2013) and 1.9 mg (Kwiri et al., 2015), but the former is within the range of the quantities presented here.

Similarly, within *Macrotermes* spp., our results are not entirely dissimilar from previously published values in some cases; yet do fall far outside the known range for some minerals. For example, Banjo et al. (2006) report 27 to 29 mg Fe content for *Macrotermes* alates, which is within the range of 12 to 40 mg detected in our three alate samples; similarly our value of 24 mg Fe for

Table 2. Mineral content (mean and range) of five edible insect species, shown alongside nutrient reference values for comparison. All data represents the mean of two replicates.

mg/100 gfw	ID	% water	Na	K	Ca	Mg	Al	P	S	Cu	Fe	Mn	Zn
RDA/AI*			1500*		1000	310		700		900	18	2*	8
Imbrasia belina	1	72	13	1436	203	151	72	565	527	3	63	5	12
	2	62	18	1864	810	312	83	780	636	4	106	7	36
	3	70	1501	1763	746	226	123	777	687	3	109	6	29
	4	93	120	940	310	110	76	410	430	1	130	5	10
Gynanisa maia	5	77	2674	1467	112	167	35	563	384	3	9	1	23
	6	74	12	1942	564	272	50	742	508	3	14	2	21
	7	93	44	1300	410	160	65	500	350	1	57	3	14
Macrotermes spp.	8	88	2086	827	136	81	54	481	237	5	19	714	15
	9	97	324	527	200	63	31	406	189	5	12	554	38
	10	91	2100	710	100	60	42	720	160	5	40	430	9
	11	74	2931	810	189	70	71	292	258	6	24	48	15
Cirina forda	12	90	970	1070	65	162	31	364	411	2	4	6	9
	13	88	481	996	113	171	34	398	459	3	6	6	29
	14	86	407	1210	91	193	35	394	422	3	6	8	12
Encosternum delegorguei	15	82	2967	440	138	101	41	372	456	5	26	1	22
	16	86	1405	227	243	95	331	348	489	28	24	2	59
	17	96	3700	280	68	83	5	340	360	3	35	2	3

N.D. indicates that the presence of this mineral was not detected in the current study. DRIs (Dietary Reference Intake) - RDA (Recommended Daily Allowance) and AI (Adequate Intake, denoted by an asterisk) - values are nutrient reference values developed by the Institute of Medicine of The National Academies and available online at:http://iom.nationalacademies.org/Activities/Nutrition/SummaryDRIs/~/media/Files/Activity%20Files/Nutrition/DRIs/5_Summary%20Table%20Tables%201-4.pdf (published online 2010; accessed 7th July 2015). Values are those recommended for women aged 19-30.

Macrotermes soldiers falls within the 10 to 55 mg range reported by Lesnik (2014). However, the Zn content in the Macrotermes soldier sample is, at 15 mg, below the 24 to 173 mg range reported by Lesnik (2014), and our three (430, 554, 714 mg) values for Mn content of alates far exceed Lesnik's (2014) range of 5 to 131 mg for mixed samples. The nutrient content of *C. forda* has also been reported on a number of occasions, and again it is clear that mineral content varies greatly between samples. Fe content, for example, ranges from 1.3 mg (Osasona and Olaofe, 2010) to 5.34 mg (Omotoso, 2006) to 64 mg (Akinnawo and Ketiku, 2000) in previously published studies, and therefore the values (4 to 6 mg) fall within this range. Similarly, the range of values for Zn content of *C forda* (9 to 27 mg) is similar to the reported values of 24.2 mg (Osasona and Olaofe, 2010), 3.81 mg (Omotoso, 2006) and 8.6 mg (Akinnawo and Ketiku, 2000), and again shows the variation in content of these micronutrients that are essential to human health. This is to our knowledge the first report of the mineral content of *G. maia,* and only the second report for *E. delegorguei.* The values for the Zn (3, 22, 59 mg) and Fe (24, 26, 35 mg) content of *E. delegorguei* are not dissimilar from the previous figures of 46 and 20.2 mg, respectively (Teffo et al., 2007), with the exception of one sample with a very low (3 mg) Zn content. Given the variation reported in other insect species, further research is required to judge whether or not this is a true outlier.

The overall results show that some edible insects contain high amounts of nutrients that are considered to be beneficial to human health, particularly Fe and Zn, when considered in relation to DRI values. *I. belina* samples, for example, contained 350 to 722% and 125 to 450% of the DRIs for Fe and Zn, respectively. If we can better understand the factors that cause variation in mineral content within species, certain edible insects may be useful candidate species for combating iron deficiency anemia. Iron deficiency anemia is a key public health problem in southern Africa, with a severe Fe deficiency of prevalence of 24.1% in one Zimbabwean study, which used blood samples from 3151 people (Sikosana et al., 1998). In South Africa a study of an unquoted number of

subjects found a 5.15 and 9 to 12% prevalence of severe Fe deficiency anemia (Nojilana et al., 2007) among children and pregnant women, respectively. Therefore, promotion of insects rich in Fe could help to combat these problems.

However, not all edible insects are alike, and some commercially available insects may contain harmful quantities of nutrients for which there is an established upper limit. This is the case for Na and Mn. High Na values are likely to be due to salt added by vendors. Our results show that the quantity of salt varies greatly between vendors and in many cases 100 g of edible insects as sold has a Na content that exceeds the DRI value of 1500 mg, and excessive consumption could therefore have negative health consequences (Appel et al., 2011). High Mn values are likely to be due to factors specific to the point of origin of the insects, such as soil and water composition. Excess manganese, when inhaled, can cause neurotoxic Parkinson-like symptoms, known as manganism (Dobson et al., 2004). Our results show that termite alates contain up to 13500% of the DRI for Mn and could therefore potentially be harmful to health if consumed in excess; however, the effect of excess dietary manganese is not clear. Overall, our results suggest that while certain commercially available indigenous edible insect foods could be beneficial in combating prevalence of iron deficiency anemia in southern Africa, others require further investigation due to potentially harmful quantities of micronutrients. Further research is required to understand the reasons for variation in mineral content within species. The unregulated and unstandardised addition of salt to edible insects sold at markets may greatly alter their nutritional profile. A final barrier to understanding the health implications of insect foods sold at markets in southern Africa is the lack of data regarding serving size: Data on average serving size for each commonly consumed insect species will be necessary in order to provide informed nutritional recommendations regarding edible insects.

Conflict of interests

The authors did not declare any conflict of interest.

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Comparative physico-chemical and proximate analysis of oils of Shea nut, *Sesamum indicum*, *Cucurbita pepo*, *Cucumis melo* seeds commonly cultivated in West Africa

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In rural areas of developing countries like Burkina Faso, nutritive elements are mainly composed of vegetable source. Shea nut, seeds of *Sesamum indicum*, *Cucumis melo* and *Cucurbita pepo,* four species widely consumed were studied. The proximate parameters: moisture, proteins and fat were analysed. Saponification value, iodine value, acid value and peroxide value of selected nut and seeds oils and fatty acids were also evaluated. The results for moisture content of oils were significantly different (P<0.05) and ranged between 3.22 ± 0.36 to 6.74 ± 0.83%. Protein rate was ranged between 12.93 ± 2.60 to 19.96 ± 0.73 with significant difference (P<0.05). Fat content was ranged at significant difference (P<0.05) as 49.14 ± 0.06, 43.82 ± 0.12, 42.01 ± 0.20 and 41.07± 0.73 for *S. indicum*, shea nut, *C. melo and C. pepo*, respectively. The acid value obtained from *S. indicum*, *C. pepo*, *C. melo* ranged between 2.51 ± 0.13, 1.29 ± 0.05 and 1.16 ± 0.06 mg/KOH/g, respectively with significant difference (P<0.05). The iodine value of the oil samples showed significant difference (P<0.05). The significant difference (P<0.05) of saponification value ranged between 197.4 ± 0.70, 191.8 ± 2.23, 117.13 ± 2.37 and 112.54 ± 0.03 from shea nut, *S. indicum*, *C. pepo* and *C. melo*, respectively. The different samples showed significant difference (P<0.05) of peroxide value ranged between 6.5 ± 0.18, 3.38 ± 0.20, 1.45 ± 0.02 and 1.33 ± 0.15 from shea nut, *S. indicum*, *C. melo, C. pepo*, respectively. The composition of fatty acids of oils revealed the presence of high amount of linoleic acid 59.12 ± 1.91 and 62.97 ± 0.62%.

Key words: Shea nut, sesame, cucurbitaceae, oil, physico-chemical, fatty acids.

INTRODUCTION

Most developing countries are confronted with protein-energy malnutrition problem in a large part of the population. Thus, while food supplies have increased between 1969-1971 and 1990-1992, the availability of

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protein and fat, decrease from 53 g / day and 30 g / day to 7 g / day (FAO/WHO, 1996). The United Nations Millennium Summit (UNICEF, 1990) made hunger eradication its first development goal (MDG), adopting the main objective of the 1996 World Food Summit; to reduce the number of undernourished people to half their present level no later than 2015 (Haddad et al., 2004). However, the United Nations Food and Agriculture Organization (FAO) estimates that 925 million persons suffer from hunger and malnutrition mainly affects Asia, South America and Africa, including 239 million people in sub-Saharan Africa (Codex, 1993b).

In Burkina Faso, a Sahelian country with about 17 million people, nearly 90% live in rural areas (UNICEF, 1990). Foods such as meat, fish and dairy products are traditional sources of protein; they are expensive and are not usually accessible to most of population.

Seeds are an alternative to protein-energy malnutrition problem due to their high fat and some protein (Bahkali et al., 1998; Egbekun and Ehieze, 1997; Rocquelin et al., 1998). It is therefore necessary to seek and diversify the sources of proteins and lipids by studying the nutritional value and the valuation of some local oil seeds such as Shea butter, sesame and some species of *Cucurbitaceae.* Shea nut fat is composed principally of triglycerides (triacylglycerols) containing an oleic acid moiety at the 2-position and saturated fatty acids, usually stearic or palmitic acids, at the 1- and 3-positions (Acquaye et al., 2001). Sesame is an important export crop in Burkina Faso and has a substantial role in the global sesame trade. The cucurbits are cultivated in different regions of the world and palatable fruits are eaten either raw (*Cucumis melo*) or cooked (*Cucurbita pepo*) with flavour. Similar to these, the seeds of cucurbits, especially pumpkin (*C. pepo* and *Cucurbita maxima*) are a common snack food in several countries and have also been used in food (Dvorkin and Song, 2002; Applequist et al., 2006).

Melon (*C. melo L.*) is a commercially important fruit crop that is cultivated worldwide (Gonzalez-Ibeas et al., 2011). It is also an important summer vegetable crop especially in the rice fallows of Kerala (Rakhi and Rajamony, 2005). Culture and use of cucurbits or squashes (*C. pepo*) have been traced to more than 10000 years ago (Idouraine et al., 1996).

The oils of *C. pepo, Brachystegia eurycoma, C. melo* and *Luffa cylindrica* seeds are generally known as nonedible oils even though some of the seeds are consumed sparingly in some localities, while that of *Arachis hypogaea* are exported on a large scale as edible oil. *C. pepo* L. produces a lot of biomass and its nutrient requirements are generally considered to be high particularly nitrogen and phosphorus (Obalum et al., 2012). Optimal mineral nutrition is fundamental to the growth and productivity of plants (Liu et al., 2010). The optimum doses of nitrogen, phosphorus and potassium vary greatly with the length of growing season, fertility status of soil, soil type, cultivar, geographical location and the environmental factors.

Seed oils are important sources of nutritional oils, industrial and pharmaceutical importance (Nzikou et al., 2010). There are numerous vegetable oils derived from various sources. These include the popular vegetable oils: the foremost oilseed oils - soybean, cottonseed, peanuts and sunflower oils; and others such as palm oil, palm kernel oil, coconut oil, castor oil, rapeseed oil and others (Nzikou et al., 2010). They also include the less commonly known oils such as *C. melo*, *C. pepo* oil and numerous others. Their yields, different compositions and by extension their physical and chemical properties determine their usefulness in various applications aside edible uses (Aluyor and Ori-Jesu, 2008). The characteristics of oil from different sources depend mainly on their composition and no oil from a single source can be suitable for all purposes (Mohammed and Jorf-Thomas, 2003).

This study was therefore undertaken to chemically analyze oils extracted from Shea nut, seed of *S. indicum, C. pepo Linn.* and *C. melo* and compare their quality with Codex standard values for food usage.

MATERIALS AND METHODS

This study was carried out in the Centre of Research in Biological, Food and Nutrition Sciences, Department of Biochemistry and Microbiology, University of Ouagadougou.

Sampling

Shea nut, was collected at Banfora (450Km of Ouagadougou) and seeds of *C. pepo*, *C. melo* and *S. indicum* were purchased at Ouagadougou (12°46'N, 129'W, altitude 301 m). The choice has been based on their availability, and their use to food ends. The plants were identified and authenticated by a Botanist at the Plants Biology Department, Ouagadougou University, Burkina Faso. Good nut and seeds were carefully selected, cleaned, shelled, well dried and crushed using laboratory Electric grinder prior to extraction.

Oil extraction

The extraction of 5.0 g of ground seed and nut was conducted in a Soxhlet extractor using n-hexane (boiling point of 40-60°C) for 6 h using adapted method of Warra et al. (2012). The oils were obtained after the solvent was shaken in reduced temperature and pressure and refluxing at 70°C to rid excess solvent used in the oil. Extracted nut and seed oil were stored in freezer at -2°C for subsequent physico-chemical analysis.

Physico-chemical analysis

Determination of moisture content

Moisture content by the oven dry method was used. Five grams of crushed sample was dried in the oven 105 ± 2°C for 5 h. The weight difference shows the moisture content (AOAC, 1990).

Determination of protein

This was measured following the Kjeldahl method based on the total mineralization of the biological material in an acid environment, followed by distillation of nitrogen in ammonia form (AOAC, 1990). The total mass of vegetable protein is calculated using a conversion factor of 6.25.

Determination of fats

A quantity of 5 g of each sample was weighed and introduced into an extraction cartridge, and covered by cotton. The cartridge was placed in a 150 ml glass Soxhlet (AOCS, 1990). The solvent container was weighed and 400 ml of n-hexane was added. The soxhlet was then introduced into the container placed on the heating mantle, which was then connected to the cryostat cooling thermostat. Four to six siphoning processes were conducted for 5 h. The heating mantle was disconnected. The solvent was evaporated in a RE 121 Rotavapor (made in Switzerland). The container with the fat was placed in an oven for 4 h at 103°C, and then in a desiccator for 30 min and weighed. The weight difference gives the fat content of the sample.

Determination of acid, iodine, peroxide and saponification value of oils extracted

The chemical analysis of the oils was carried out using the methods reported as AOAC, 1998; Akpan et al., 2006.

Acid value: A volume of 100 ml of neutral ethyl alcohol was heated with 10 g of oil or fat sample in a 250 cm^3 beaker until the mixture began to boil. The heat was removed and was titrated with N/10 KOH solution, using two drops of phenolphthalein as indicator with consistent shaking for which a permanent pink colour was obtained at the end point.

The Acid value was calculated using the expression;

$$AV = 0.56 \times \text{No. of ml. N/10 KOH used}$$

Iodine value: A Quantity of 0.4 g of the sample was weighed into a conical flask and 20 cm^3 of carbon tetra chloride was added to dissolve the oil. Then 25 cm^3 of Dam's reagent was added to the flask using a safety pipette in fume chamber. Stopper was then inserted and the content of the flask was vigorously swirled. The flask was then placed in the dark for 2 h 30 min. At the end of this period, 20 cm^3 of 10% aqueous potassium iodide and 125 cm^3 of water were added using a measuring cylinder. The content was titrated with 0.1 M sodium thiosulphate solutions until the yellow color almost disappeared.

Few drops of 1% starch indicator was added and the titration continued by adding thiosulphate drop wise until blue coloration disappeared after vigorous shaking. The same procedure was used for blank test and other samples. The iodine value (I.V) is given by the expression:

$$IV = 12.69C (V_1 - V_2)/M$$

Where, C = Concentration of sodium; V_1 = volume of sodium thiosulphate used for blank; V_2 = volume of sodium thiosulphate used for determination and M = mass of the sample.

Saponification value: A quantity of 2 g of the oil sample was added to a flask with 30 cm^3 of ethanolic KOH and was then attached to a condenser for 30 min to ensure the sample was fully dissolved.

After sample had cooled, 1 cm^3 of phenolphthalein was added and titrated with 0.5 M HCl until a pink endpoint has reached. Saponification value was calculated from the equation:

$$SV = (S-B) \times M \times 56.1/\text{Sample weight (g)}$$

Where, S = sample titre value; B = blank titre value; M = molarity of the Hcl; 56.1 = molecular weight of KOH

Peroxide value: Peroxide value has been determined according to the AOCS methods, 1990 (AOAC 965.33).

Determination of the composition in fatty acids by the gas-liquid chromatography

The samples were previously mixed with methyl to the proportion of 200 mg of lipids for 10 ml of the hydrochloric methanol mixture (25 ml of chloride of acetyl in 250 ml of methanol). After dilution, the fat phase was extracted with 20 ml of hexane, then washed until neutrality, concentrated by evaporation and dried to the sulphate of sodium.

The prepared samples were injected into the chromatograph to ionize the flame marks Girdel 30, provided with a column carbowax 20 M and with a recording Shumadzu CR4A. The methylic esters were identified by comparison with a chromatogram standard achieved with fatty acids of reference. The conditions of analysis were as follows: Temperature of the oven: 180°C; Temperature of the injector: 220°C; temperature of the detector: 260°C; vector gas: nitrogen; pressure of entry of the vector gas: 2.5 bars; debit of the vector gas: 1.65 ml/min; debit of the gas of food of the detector to ionization of flame (FID): air: 75.5 ml/min. hydrogen: 8.5 ml/min.

Statistical analysis

All experiments were conducted in triplicate and the statistical significance differences of mean were calculated using SAS (20.1), with the help of one-way ANOVA. Results are expressed as means ± SD. A probability value at $p<0.05$ was considered to denote the statistically significant differences.

RESULTS AND DISCUSSION

The results obtained from the experimental work are presented in Tables 1 and 2. The tables include results of as well as the physical and chemical compounds of the extracted oil as well as the fatty acid profile.

The results in table 1 showed that moisture content of oils were significantly different ($P<0.05$) and ranged between 6.74 ± 0.83, 4.6 ± 0.11, 4.32 ± 0.37 and 3.22 ± 0.36% shea nut, *C. pepo*, *S. indicum*, *C. melo* respectively. They were very high, far exceeding that of *A. hypogaea* oil (0.089%) and also the stipulated Codex standard (0.05%). This high moisture content creates problem in trans esterification.

Protein rate ranged between 12.93 ± 2.60 and 19.96 ± 0.73 with significant difference ($P<0.05$). In the seed of *S. indicum*, protein was higher than all seeds and nuts. This indicates that these could be used for food enrichment and against malnourishment.

Table 1. Physicochemical characteristics of the oils of shea nut, *Sesamum indicum*, *Cucurbita pepo*, *Cucumis melo* seeds.

Parameter	Shea nut	*Sesamum indicum*	*Cucurbita pepo*	*Cucumis melodrama*
Moisture (g/100 g)	6.74 ± 0.83[a]	4.32 ± 0.37[b]	4.6 ± 0.11[b]	3.22 ± 0.36[c]
Protein (g/100 g)	12.93 ± 2.60[a]	19.96 ± 0.73[b]	18.87 ± 1.50[c]	18.60 ± 0.02[c]
Fat (g/100 g)	43.82 ± 0.12[a]	49.14 ± 0.06[b]	41.07 ± 0.73[c]	42.01 ± 0.20[c]
Acid value (mgKOH/g)	11.17 ± 1.62[a]	1.16 ± 0.06[b]	1.29 ± 0.05[b]	2.51 ± 0.13[c]
Iodine value (mg I2/100 g)	54.14 ± 1.19[a]	108.28 ± 3.11[b]	95.55 ± 0.16[c]	76.34 ± 2.6[d]
Saponification value (mgKOH/g)	197.4 ± 0.70[a]	191.8 ± 2.23[b]	117.13 ± 2.37[c]	112.54 ± 0.03[d]
Peroxide value (meqO_2/kg)	6.5 ± 0.18[a]	3.38 ± 0.20[b]	1.33 ± 0.15[c]	1.45 ± 0.02[c]

Means with different letters on row are significantly different at P = 0.05.

Table 2. Fatty acid composition (g/100g) of oils extracted from four plants.

Free fatty acid	Shea nut	*Sesamum indicum*	*Cucurbita pepo*	*Cucumis melo*
Palmitic acid C16 : 0	3.65 ± 0.28[a]	11.92 ± 0.58[b]	10.50 ± 0.70[b]	8.18 ± 0.49[c]
Palmitoleic acid C16 : 1	-	0.23 ± 0.12	-	-
Stearic acid C18 : 0	42.87 ± 0.7[a]	5.18 ± 0.20[b]	8.89 ± 0.73[c]	12.43 ± 1.00[d]
Oleic acid C18 : 1	42.91 ± 1.2[a]	41.20 ± 1.13[a]	17.22 ± 1.12[b]	18.11 ± 0.74[b]
Linoleic acid C18 : 2	6.85 ± 0.20[a]	37.37 ± 0.88[b]	62.97 ± 0.62[c]	59.12 ± 1.91[c]
Ratio unsaturated/saturated	1.13[a]	4.60[b]	4.15[b]	3.75[c]

Means with different letters on row are significantly different at P = 0.05.

Fat content ranged with significant difference (P<0.05) as 49.14 ± 0.06, 43.82± 0.12, 42.01 ± 0.20 and 41.07 ± 0.73 for *S. indicum*, Shea nut, *C. melo, C. pepo* respectively.

An Acid value was ranged from 1.16 ± 0.06 to 11.17 ± 1.62 mgKOH/g with significant difference (P<0.05). Highest value was obtained in oil of the Shea nut (11.17 ± 1.62 mgKOH/g) which is lower than that of olive oil 17 mgKOH/g (Oyedele, 2006), higher than Codex Stan 19-(1993a) acceptable value (4 mg/KOH/g).

The acid values obtained from *S. indicum*, *C. pepo*, *C. melo* ranged between 2.51 ± 0.13, 1.29 ± 0.05 and 1.16 ± 0.06 mg/KOH/g; were lower than Codex STAN 19-(1993a) limit value (4 mg/KOH/g) and those obtained by Eka and Chidi (2009) for butternut oil and Akubugwo and Ugbogu (2007) for African star apple oil which reported acid values of 4 for sesame, soybean, sunflower and rape seed and 7 for olive oil.

Acid value is a direct measure of the percentage content of free fatty acids in a given amount of oil. It is a measure of the extent to which the triglycerides in the oil have been decomposed by lipase action into free fatty acids; acid value depends on the degree of rancidity which is used as an index of freshness (Ochigbo and Paiko, 2011). It is common knowledge that these parameters are a measure of the level of spoilage of oil, hence we conclude that they are of low magnitude and a reflection of the freshness and edibility of the crude oil.

The iodine value of oil of samples showed significant difference (P<0.05). The values obtained on extracted oils of *S. indicum*, *C. pepo*, *C. melo* and Shea nut were 108.28 ± 3.11, 95.55 ± 0.16, 76.34 ± 2.6 and 54.14 ± 1.19 mg I_2/100 g respectively. *S. indicum* iodine value was in agreement with critical value (104-120 mg I2/100 g) of Codex Stan 26 (1993a). All of samples were higher than 31.06 ± 0.80 mg/100 g found from previous work on African star apple seed by Akubugwo and Ugbogwu (2007). Oils are classified into drying, semi-drying and non-drying according to their iodine values. The iodine value of *C. pepo*, *C. melo* seeds, Shea nut oil is lower than 100, it could only be classified as a non-drying oil. The low iodine value indicates that the oil has a low content of unsaturated fatty acids thus resembles olive oil and groundnut oil, could be employed for food and other use (Dosunmu and Ochu, 1995).

The significant difference (P<0.05) of saponification value ranged between 197.4 ± 0.70; 191.8 ± 2.23, 117.13 ± 2.37 and 112.54 ± 0.03 from Shea nut, *S. indicum*, *C. pepo* and *C. melo* respectively.

Shea nut and *S. indicum* compared favourably with values obtained by Mohammed and Hamza (2008) for sesame seed (189 to 190 mg/KOH/g) and some common oils like palm oil (196-205 mg/KOH/g), groundnut oil (188-196 mg/KOH/g), corn oil (187-196 mg/KOH/g) also Codex Stan, 1993a (187-195 mg KOH/g). They were lower than that of coconut oil (253 mg/KOH/g) and palm kernel oil (247 mg/KOH/g). According to Ezeagu et al. (1998) a saponification value of 200 mg KOH/g indicates

high proportion of fatty acids of low molecular weight. This shows that the oil may have a potential for use in soap making and cosmetics industry and for the thermal stabilization of poly vinyl chloride (PVC). These properties make them useful as sources of essential fatty acids required in the body (Akanni et al., 2005). However, saponification values obtained are within the range for edible oils reported by Eromosele et al. (1994).

The different samples showed significant difference (P<0.05) of peroxide value ranging between 6.5 ± 0.18, 3.38 ± 0.20, 1.45 ± 0.02 and 1.33 ± 0.15 from *Shea nut, S. indicum*, *C. melo, C. pepo* respectively. These values obtained were lower than limited value (10 meq/Kg) of Codex Stan 19, (1993a). Peroxide value is an index of rancidity, thus the high peroxide value of oil indicates a poor resistance of the oil to peroxidation during storage (Mohammed and Hamza, 2008). The peroxide values of African star apple seeds are 1.57 meq/KOH/g which is below the maximum acceptable value of 10 meq/KOH/g set by the Codex Alimentarius Commission for such oils as groundnut seed oils (Abayeh et al., 1998). *C. melo* and *C. pepo* were in agreement with the seed oil of cultivated cucurbits from Egypt contained 3.21-3.60 meq/kg of peroxide value (El-Adawy and Taha, 2001). Peroxide value is an indication of deterioration of oil level. The low peroxide value further confirms the stability of the oil. Fresh oils have values less than 10meq/kg. Higher values between 20 and 40 results to a rancid taste (Akubugwo and Ugbogu, 2007). The low acid and peroxide values are indicators of the ability of the oil to resist lypolitic hydrolysis and oxidative deterioration (Akanni et al., 2005).

Fatty acids composition of four plants seed and nut oils as analyzed by gas chromatography is presented in Table 2. Palmitic acid was found significantly different (P<0.05) according to the oils source and ranged from 3.65 ± 0.28 to 11.92 ± 0.58%. Recommended value of Codex Stan 26 (1993a) is 7-12%. *S. indicum* expressed the highest percentage palmitic acid and contained also palmitoleic acid as 0.23 ± 0.12% and was lower than codex stan 26 (1993a) value (<0.25%). Stearic acid detected with significant different (P<0.05) in the tested oils was 42.87 ±0.7, 12.43 ± 1.00, 8.89 ± 0.73 and 5.18 ± 0.20 from Shea nut*, C. melo C. pepo* and *S. indicum* respectively. Recommended value of Codex Stan 26 (1993a) is 3.5 -6.0%. The range of oleic acid and linoleic acid were 17.22 ± 1.12% to 42.91 ± 1.2% and 6.85 ± 0.20 to 62.97 ± 0.62 with significant difference (P<0.05) between the oils. *C. melo and C. pepo* expressed the highest percentage of linoleic acid. Ratio unsaturated/ saturated showed higher value (4.60) has been observed in oil of *S. indicum seed.* Previously, Sew et al. (2010) also found that winter melon seed oil had linoleic acid (67.37%) as the principal component, followed by palmitic acid (17.11%), oleic acid (10.21%) and stearic acid (4.83%), respectively. *C. melo and C. pepo* seed oils could be explored as a potential source of omega 6 dietary supplements.

The concentration of major fatty acids as studied in the present work could be comparable with other cucurbits seed oils. *Cucumeropsis manni* seed oil had a range of 15-24, 10-12.3, 9-18 and 42-61% of palmitic acid, stearic acid, oleic acid and linoleic acid, respectively (Badifu, 1991; Fokou et al., 2009). *C. pepo* seed oil contained fatty acids compound within a range of 9.9-49.2% (palmitic acid), 4.87-11.2% (stearic acid), 17.0-47.0% (oleic acid), and 4.9-55.6% (linoleic acid) (Tsaknis et al., 1997, El-Adawy and Taha, 2001, Nakic et al., 2006; Nyam et al., 2009). Palmitic acid (10.7-11.36%), stearic acid (7.04-9.00%), oleic acid (13.25-18.1%) and linoleic acid (59.6-68.3%) were detected as the most abundant fatty acids in *Citrullus lanatus* seed oils (El-Adawy and Taha, 2001; Milovanovic and Picuric-Jovanovic, 2005; Mariod et al., 2009, Nyam et al., 2009; Baboli and Safe Kordi, 2010).

Conclusion

The extracted seed oils revealed the presence of high amount of linoleic acid (59.12 -62.97%) in *C. pepo* and *C. melo,* placing these oils in the category of high-linoleic vegetable oils. The proximate analysis of seeds and physicochemical attributes of the extracted seed oils were appraised for the four grown in West Africa. The seeds and nut from the tested plants were explored as a good source of oil, protein and thus could be consumed for dietary purposes. Further investigation on tocopherol and phytosterol amount of the seed and nut oils is strongly recommended.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Comparative study of bioethanol production from sugarcane molasses by using *Zymomonas mobilis* and *Saccharomyces cerevisiae*

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The study was designed to compare the bioethanol production from *Zymomonas mobilis* and *Saccharomyces cerevisiae* using molasses as production medium. The focus was on the retention time at lab scale. Bioethanol and petroleum blend can be used in existing gasoline engines. Present study showed a more cost-effective procedure for production of ethanol from sugar-cane molasses by using bacterial strain "*Z. mobilis*". Laboratory scale unit was designed to perform the experiments through batch fermentation and to determine the impact of leading parameters, including fermentation temperature, pH, sugar concentration, and nutrients. *S. cerevisiae* produced 8.3% (v/v) bioethanol provided sugar concentration 14 g /100 ml with the fermentation efficiency of 92.5%. On the contrary, *Z. mobilis* produced 9.3% (v/v) bioethanol by utilizing 16 g/100 ml sugar with the fermentation efficiency of 90.5%. Effect of nutrients on fermentation was determined using molasses as feedstock. Thin layer chromatography was also performed to assess the possible impurities in molasses as compared to the pure sugar. The pH and fermentation temperature was optimized for the enhanced yield of bioethanol.

Key words: Bioethanol, molasses, fermentation, *Zymomonas mobilis, Saccharomyces cerevisiae*.

INTRODUCTION

Nowadays, petroleum products are running out of race due to unbalanced relation between supply and demand, also escalations in the oil prices for the last two decades are contributing to set trends for the use of alternative resources. Pakistan imports million tons of oil every year to meet their energy constraint (US Department of Energy, 2014). Referable to the current scenario of energy, Pakistan needs to pay special attention to

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alternative fuels using feedstock like biomass and surplus molasses which are cheaper source of energy in some developing countries. Among the biofuels, bioethanol is very impressive and leading fuel produced in the different parts of the world (Mousdale, 2011). The literature records that the bioethanol usage cause low emissions of greenhouse gases (GHG) (Lee and Shah, 2012). Ethanol can be produced by utilizing the biomass, molasses, or any lignocellulosic material with the help of micro-organisms. In this study, the sugar industry molasses was used as feedstock, which is widely available in sugar producing regions (Ayhan, 2008). Pakistan is producing 2 to 2.5 million tons of molasses every year, and 80% of molasses are being exported every year (PMSA, 2013) and if it is locally used in Pakistan for the production of bioethanol, it may be able to harvest 2 to 3% transportation fuel annually (Ali, 2013). Molasses is a byproduct of the sugar industry, has a significant quantity of sugar 40 to 50% (w/v), ash content of 5 to 15%, which is used as a substrate in the rum and bioethanol production from many years (Doelle and Doelle, 1990). The utilization of sugar cane molasses for the treatment method such as fermentation which is one of the oldest chemical processes known to human and most widely practiced by them is used to produce a variety of valuable chemicals (Mousdale, 2011).

In recent years, however, many of the products are synthesized cost effectively from petroleum feedstock, including bioethanol. The use of microorganisms is usually considered as environment friendly. The efficiency and specificity of the microorganism are an advantageous aspect to produce targeted products like bioethanol (Balat et al., 2008). From last three decades, studies were carried out to minimize the issues in the fermentation technology for efficient bioethanol production (Balat et al., 2008; Bulock et al., 1984). *Saccharomyces cerevisiae* (*S. cerevisiae*) was used widely in commercial scale, but *Zymomonas mobilis* (*Z. mobilis*) was not commercially used currently due to some constraints. Sadik et al. (2014) has reported that *Z. mobilis* has some advantages over *S. cerevisiae* with respect to time required for the completion of the fermentation process with targeted yield (Belkis et al., 1998). Diverse studies were conducted to sort out the issues in fermentation process by using yeast and bacteria (Banks and Aswad, 2013). In this study, the factors effecting the bioethanol production has been investigated for the optimum yield of bioethanol in prime conditions using molasses from local sugar mills in Pakistan.

MATERIALS AND METHODS

Material and culture

Sugar cane molasses were provided by Noon Sugar mills Pvt. Limited Bhulwal Pakistan. The two strains used in the study were *Z. mobilis* and *S. cerevisiae*. *Z. mobilis* was purchased by DSMZ Germany and a local dealer in Islamabad provided the yeast strain. The media for maintaining *Z. mobilis* culture as suggested by DSMZ Germany (2013) contain bacto-peptone (10 g/L), yeast extract, 10; 15 g/L agar for agar plates, glucose (20 g/L) with pH 7.00. Media was autoclaved at 121°C and 15 psi, for 20 min. To obtain single bacterial colonies, bacterial cells were isolated on the Petri plates by standard streak plate method under sterilized conditions and incubated at 37°C for overnight. Bacterial cells were characterized by Dichromate test and Potassium permagnate tests. The cells were inoculated in 5 ml sterilized Luria Bertani (LB) media. Number of cells were calculated by hemocytometer. *S. cerevisiae* was grown in YNPG media (Bergman, 2001) containing yeast extract (5 g/L), peptone (10 g/L), NaCl (10 g/L), glucose (10 g /L), Agar (15 g/L) for agar plates at pH 7.00. The media was sterilized at 121°C, 15 psi for 20 min. Yeast culture was streaked on YNPG agar plates by Streak Plate method and incubated at 37°C for overnight. Inoculation into test tubes for scale up and cell count was conducted. *S. cerevisiae* was maintained in glycerol stock for further use.

Analysis for total sugar concentration and impurities in molasses

Sugar concentration in molasses was determined through Fehling's test (Thorpe, 2002). Five (5) mL molasses sample was dissolved in 100 ml of distilled water and 5 ml of concentrated HCl was added to it, and heated at 70°C for 10 min. 1 M NaOH solution was taken in burette; added into the solution till neutralized; then the burette reading was recorded; its titrated value (TV). Five (5) mL of Fehling A and Fehling B solution was taken, mixed with 10 ml of distilled water in a conical flask and methylene blue indicator was added. Solution was titrated with a burette solution in boiling conditions until the disappearance of blue color and the burette reading notified as Fehling factor (FF). Fehling's "A" contains 7 g $CuSO4.5H_2O$ dissolved in distilled water containing 2 drops of dilute sulfuric acid. Fehling's "B" contains 35 g of potassium tartrate and 12 g of NaOH in 100 ml of distilled water. The sugar concentration was calculated by following formula:

$$TS = \left[\frac{DF \times FF}{TV}\right] \times 100 \quad (1)$$

TS = Total sugars, DF = Dilution factor (Dilution of molasses for sugar concentration), FF = Fehling factor, TV = Titrate value, Total sugar concentration was also determined by a second method using portable Refractometer RHB 32ATC, Japan. Total sugar found out to be 47%.

Impurities were analyzed by Thin Layer Chromatography (TLC) using water and hexane as a solvent. Two homogenous solvent chambers were maintained, one containing water as solvent and other containing hexane as solvent. Two spots of solution containing molasses were placed on two separate silica gel coated plates, about 1.5 cm from the bottom edge, allowed to dry completely and then placed in the chambers maintained with solvents. The solvent moved up the plate by capillary action, met the molasses mixture, and carries the soluble constituents up the plate. Then, the plates were removed from the chambers before the solvent front reaches the top of the stationary phase and dried.

Development of molasses inoculum for batch fermentation

One hundred (100) ml of concentrated molasses containing the sugar concentration of 7 g/100 ml were taken in a conical flask and inoculated with 5 ml of overnight grown inoculum of bacteria and yeast separately. The whole assembly was placed in incubator set

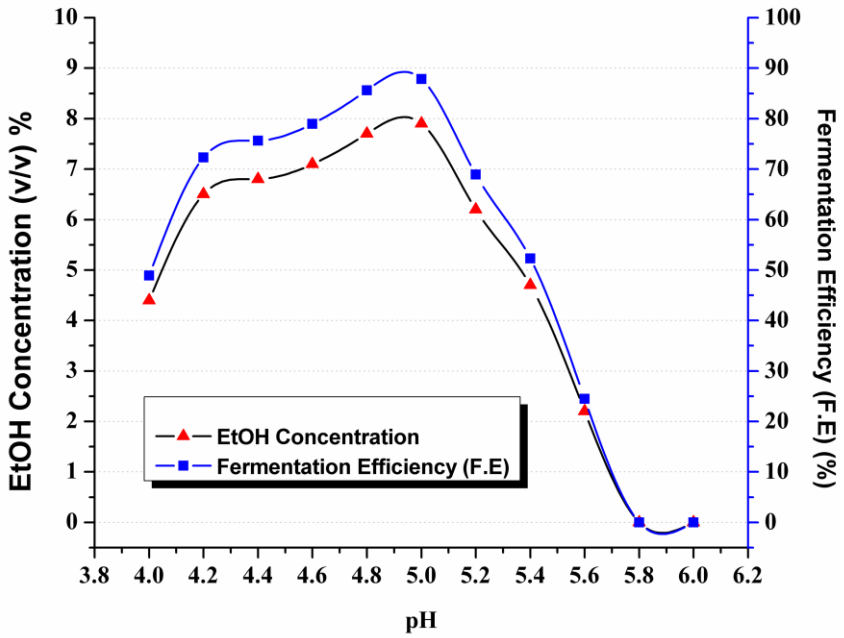

Figure 1. Effect of pH for bacterial (*Z. mobilis*) fermentation.

at 37°C overnight. Cell counts were conducted for both samples by hemocytometer; desirable growth was achieved and used for fermentation.

Batch fermentation

The known amount of sugar cane molasses and growth media was taken in fermentation flask, was inoculated and kept in shaker for fermentation. Anaerobic conditions were maintained for two days, and strains converted sugar into bioethanol with the evolution of CO_2. Samples were tested after 48 h, similar techniques were applied to *S. cerevisiae* to investigate the effect of sugar concentration, pH, fermentation temperature, supply of nutrients and effect of impurities in molasses.

Identification of bioethanol

Five (5) ml fermented sample was taken and pinch of Potassium dichromate, and few drops of concentrated H_2SO_4 were added. The brownish color of sample was changed into green which indicated the presence of bioethanol.

Determination of bioethanol concentration and pH

Ebulliometer (J. SALLERON DUJARDIN Sr. PARIS) which was approved in distilleries (US Department of Commerce, 1974) determined the bioethanol concentration, based on volatility. The reference temperature has been recorded for water, which was used in the process. The pH was determined by pH meter handy (EZDO 6011 Japan).

Acidity test

Ten (10) ml of fermented sample was taken in beaker. The beaker was put in the stirrer and its pH was checked. The sample was taken in a burette, titrated the sample by 1 N NaOH solution until its pH reached to 7.00. The reading was noted and multiplied by 0.69, which are equivalent weight of sulphuric acid. The output obtained was acidity.

Fermentation efficiency

The fermentation efficiency was calculated by the given formula:

FE = (actual yield **/** theoretical yield) ×100

FE = fermentation efficiency.

RESULTS AND DISCUSSION

Effect on pH

To obtain maximum yield of bioethanol, samples were fermented in different pH ranges from 4.0 to 6.0. The sugar concentration, cell density, and temperature were kept constant. Anaerobic conditions have been applied, and the fermented samples were analyzed after 48 h as reported (Hadiyantoa et al., 2014). Figure 1 shows the result for *Z. mobilis*, maximum yield 7.9 (v/v) has been achieved in range of 5.0 pH with the fermentation efficiency of 88%. Adjustment of the pH using acid/base may cause the lower yield and production of acids that has been affirmed by acidity test however, maximum productivity was observed at 5.0 to 5.5 pH (Doelle and Doelle, 1990; Yanase et al., 2005). On the other side, the same parameters were set for *S. cerevisiae* fermentation as well and found the trends as shown in Figure 2. It

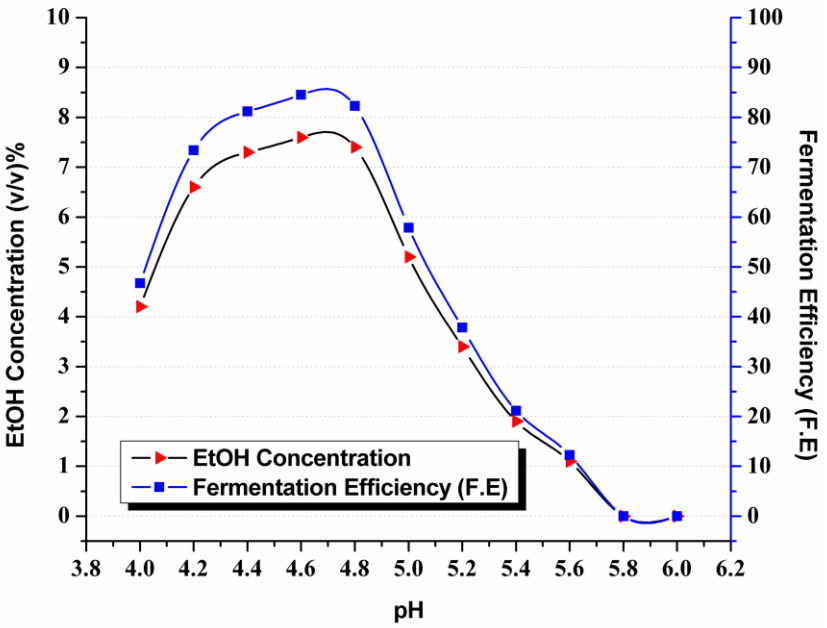

Figure 2. Effect of pH for yeast (*S. cerevisiae*) fermentation.

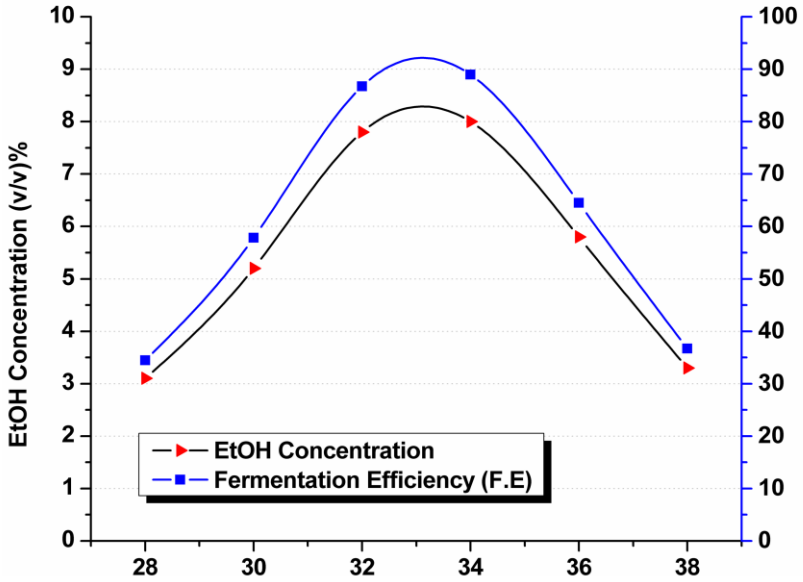

Figure 3. Effect of fermentation temperature (*Z. mobilis*).

shows that increasing pH causes increase in bioethanol yield until pH 4.6, further increase in pH cause decreases in bioethanol yield. A yield of 7.6(v/v) was achieved in 4.6 pH with the fermentation efficiency of 88%. It is clear from above assessment that the *Z. mobilis* can produce optimal yield in the high pH as compared to *S. cerevisiae*. The currents studies also reveal that the optimum pH for yeast is in the range of 4.0 to 4.6 (Hemamalini et al., 2012) (Nigam, 1999). The fermentation time was also investigated and it was recorded that the optimum yield of bioethanol was achieved at 30 to 36 h of incubation for *Z. mobilis* and 48 to 60 h for *S. cerevisiae.*

Effect of fermentation temperature

Samples were maintained in the optimum pH 5.0 for *Z. mobilis* and pH 4.6 for *S. cerevisiae* with the temperature range from 28, 30, 32, 34, 36 and 38°C as shown in Figures 3 and 4. The samples were fermented for 48 h; after analyzing the samples it found that the optimum yield was achieved in 34°C for *Z. mobilis* where the

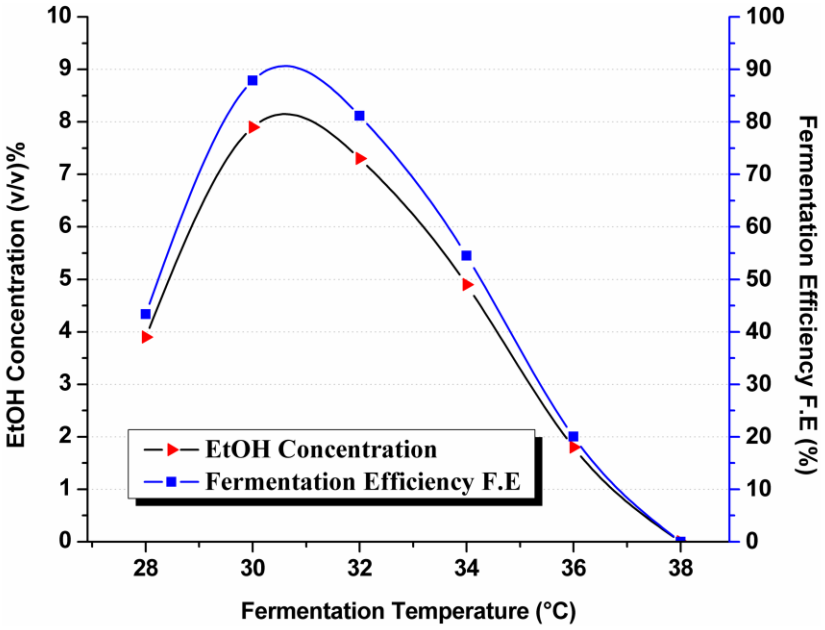

Figure 4. Effect of fermentation temperature (*S. cerevisiae*).

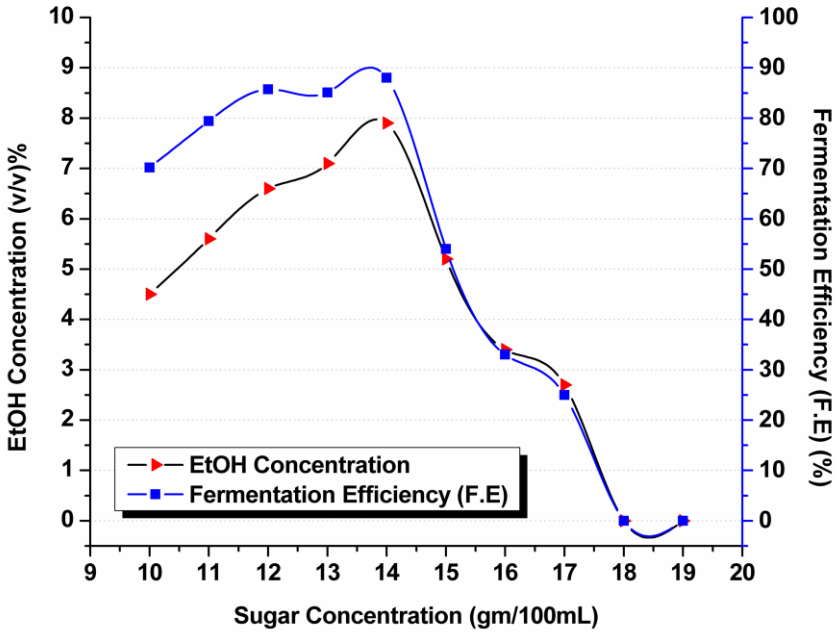

Figure 5. Effect of sugar concentration on yeast (*S. cerevisiae*) fermentation.

bioethanol yield was 8.0% (v/v) with the fermentation efficiency of 88.96%. By increasing temperature, increased bioethanol yield was observed until 34°C. Furthermore, the yield decreases by further increasing fermentation temperature. The effect of temperature for bacterial fermentation was studied before by Doelle and Doelle (1990) and Panesar et al. (2007); they recorded 35°C as optimum temperature. Similarly, the *S. cerevisiae* showed, the maximum yield of 7.9%(v/v) at 30°C with fermentation efficiency of 87.85% as shown in Figure 5. The Figure reflects that the temperature is a very sensitive parameter for *S. cerevisiae* because it produced maximum yield at 30°C but bioethanol production declined at higher temperature due to the

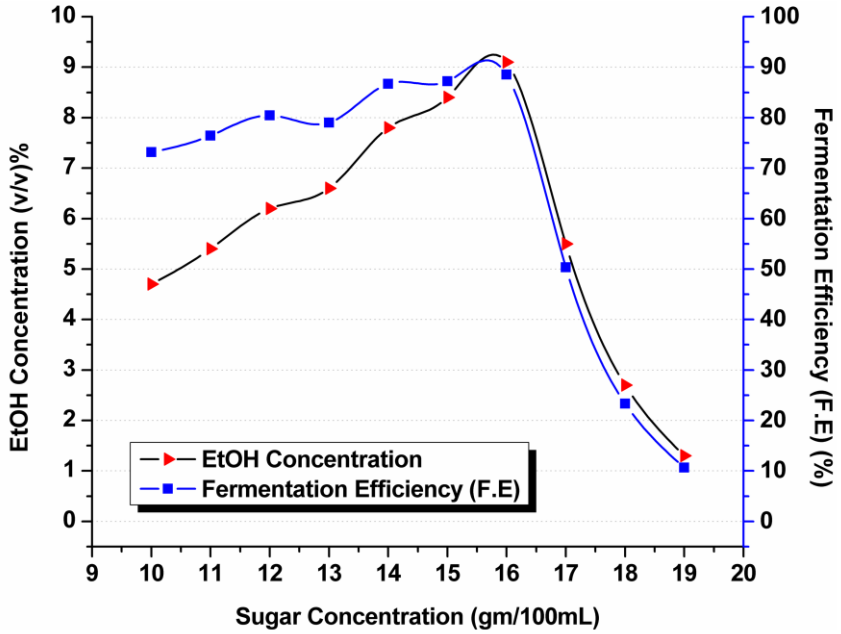

Figure 6. Effect of sugar concentration on bacterial (*Z. mobilis*) fermentation.

denaturation of *S. cerevisiae* cells. *S. cerevisiae* was unable to tolerate the elevated temperature, due to this factor in summer; the yield of bioethanol production is quite low in distilleries. On the contrary, the *Z. mobilis* produced the maximum yield in high temperature. From these results, it was observed that the *S. cerevisiae* was suitable for low temperature process while *Z. mobilis* can be used in regions having an elevated-temperature process. Kopsahelis et al. (2007) studied the effect of fermentation temperature and found 30°C as optimum in case of yeast fermentation. Kirk and Aswad (2013) followed the same method and found that the optimum yield can be obtained at 32°C for yeast fermentation though exothermic reaction values may differ from process to process because of rise in temperature during fermentation (Table 2).

Effect of sugarcane molasses concentration

Sugar molasses concentrations were varied from 10 g/ to 18 g/100 ml for both *S. cerevisiae* and *Z. mobilis*, keeping the pH and temperature constant (pre-optimized). In the case of bacteria (Figure 6) increasing sugar concentration caused the enhanced bioethanol productivity and reached optimum at 16 g/100 mL with 8.5% (w/v) with the fermentation efficiency of 81%, after the yield goes down by increasing concentration.

Effect of nutrients

Nutrients are quite effective in the production of bioethanol from sugar cane molasses (Fadel et al., 2013; Cazetta et al., 2007). Di-Ammonium phosphate (DAP) and urea was supplied to the fermentation with pre-optimum parameters. The contribution of the DAP and urea in the process was 1:1. Different nutrients can be supplied for the fermentation process, the most effective nutrients found are DAP and urea reported by Fadel et al. (2013). However, their quantity was adjusted according to set parameters in experiment. Yield increased with the addition of nutrients in the fermentation process with a notable efficiency in case of *S. cerevisiae.* Table 1 show that the 2 gm /L was found as best quantity to get the optimum yield of 9.30%(v/v) with the fermentation efficiency 90.5% for *Z. mobilis* while, yeast produces 8.3%(v/v) with the fermentation efficiency of 92.3%, which is considered as more yield than bacteria. Using nutrients as supplement, the *S. cerevisiae* showed more increments in the yield as compared to *Z. mobilis*.

Effect of impurities in fermentation

To check the effect of impurities in fermentation, an experiment was conducted, in pre-optimized conditions, which were already conducted and results were observed. Using pure sugar as a substrate instead of molasses, (Table 3) results were affirmative. Thin Layer Chromatography (TLC) analyzed the impurities and find whether there are any impurities that inhibited the bioethanol production, and suppress the activity of enzymes (Cazetta et al., 2007). While, utilizing pure sugar the bioethanol yield for *S. cerevisiae* is 8.6% (v/v) with fermentation efficiency of 95.5%. *Z. mobilis*

Table 1. Effect of nutrients (DAP+Urea) in bacterial fermentation.

DAP+Urea (g/L)	p.H	Sugar conc. (g/100 ml)	F. temp°C	Acidity	EtOH Yield (v/v)	F.E %
1	5	16	33	4.4	8.9	86.6
2	5	16	33	4.1	9.3	90.5
3	5	16	33	4.2	0.1	88.5

Table 2. Effect of nutrients (DAP+Urea) in yeast fermentation.

DAP+Urea (g/L)	p.H	Sugar conc. (g/100 ml)	F. temp°C	Acidity	EtOH Yield (v/v)	F.E %
1	4.6	14	30	4	8.3	92.3
2	4.6	14	30	5.1	7.9	87.9
3	4.6	14	30	4.7	8	88.9

Table 3. Effect of impurities in fermentation.

Microorganism	Nutrients (g/L)	p.H	Sugar conc. (g/100 ml)	F. temp °C	Acidity	EtOH Yield (v/v)	F.E %
S. cerevisiae	1	4.6	14	30	4.1	8.6	95.5
Z. mobilis	2	5	16	33	4.2	9.6	93

Table 4. Effect of fermentation time.

Microorganisms	Concentration of EtOH (v/v)	Fermentation time (h)	F.E (%)
S. cerevisiae	8.67	52	95.6
Z. mobilis	9.59	33	93

produced 9.6%(v/v) with the efficiency of 93%. It can be concluded that impurities in molasses may influence on enzymatic activity and yield can be enhanced using some enzyme stabilizers or some agents/additives, which may nullify the effects of impurities. Thin layer chromatography of molasses also reveals that the impurities are water-soluble only and cannot be dissolved in hexane.

Effect of fermentation time

For optimum yield, the fermentation time found was given in Table 4 while applying the pre-optimized parameters.

Conclusion

The optimized conditions were found by analyzing different parameters for *Z. mobilis* and *S. cerevisae.* The optimum condition for bacteria was recorded as 9.3%(v/v); bioethanol can be produced with efficiency of 90.5%, at sugar concentration of 16 g/100 mL, pH 5.0 and fermentation temperature of 34°C. Two (2) g/L nutrients (DAP, urea) were supplied to get the optimum yield. For yeast, it was found that optimum bioethanol yield like 8.3%(v/v) can be obtained at pH 4.6, sugar concentration of 14 g/100 ml, fermentation efficiency of 92.3% and fermentation temperature of 30°C. One (1) g/L nutrients (DAP, urea) were supplied in the same ratio. The impurities in the molasses are also responsible for the lesser bioethanol yield in fermentation process. The fermentation time was investigated while keeping other parameters in optimized condition.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Silica gel matrix immobilized *Chlorophyta hydrodictyon africanum* for the removal of methylene blue from aqueous solutions: Equilibrium and kinetic studies

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Chlorophyta hydrodictyon africanum **was immobilized on a silica gel matrix to improve its mechanical properties. The algae-silica gel adsorbent was used for batch sorption studies of a cationic dye, methylene blue (MB). Optimum adsorption was obtained with a dosage of 0.8 g bio sorbent. Results from sorption studies show that 124.11 mg·g^{-1} of MB could be adsorbed at an optimum pH of 8 and immobilization of 300 mg per gram silica. Maximum immobilization was 400 mg biomass per gram silica. Sorption capacity increased with an increase in initial dye concentration and reached equilibrium within 30 min. Three models were used to simulate kinetic data and the pseudo–second order model gave a better fit with R^2 greater than 0.98 in all cases. Equilibrium studies revealed that the adsorption of MB followed Freundlich isotherm (R^2=1.00).**

Key words: Adsorbent, algae, Langmuir model, Freundlich isotherm.

INTRODUCTION

The ever growing population and industrialization has led to environmental disorder as large numbers of xenobiotic compounds are being accumulated (Khataee et al., 2013). Dye effluent from the textile, pulp and paper industries is one of the major environmental concerns from a toxicological perspective (Ahmaruzzaman, 2009). The industries use dyes and pigments to color their products. Mane et al. (2007) reported that the colored effluent from these industries is a dramatic source of aesthetic pollution and perturbation of aquatic life. According to Namasivayam et al. (2001) and Waranusantigul et al. (2003), dye effluents in receiving streams interfere with transmission of light into streams and reduce photosynthesis. Many dye compounds and their metabolites are either, toxic, carcinogenic or teratogenic (Gong et al., 2007).

Removal of low concentrations of organic and inorganic substances from industrial effluent has encountered both technical and economic challenges. Although there are traditional methods of treating industrial effluent, these methods have challenges of their own and hence there has been a continual research in to more economic and environmentally friendly methods (Srinivasan et al., 2010; Gupta et al., 2009; Kyzas and Matis, 2013). One of the approaches that have attracted a lot of research interest has been the development of bio-adsorbents based on

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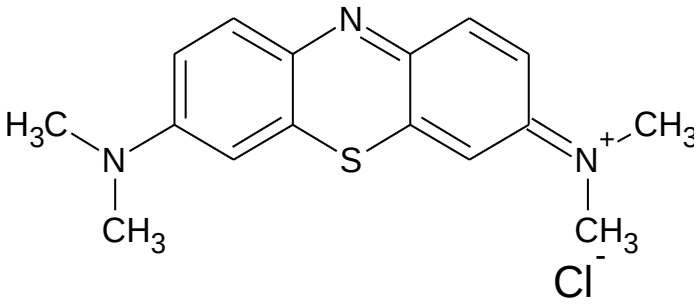

Figure 1. Chemical structure of methylene blue.

Table 1. Methylene Blue biosorption using selected alga species.

Algal species	Maximum adsorption ($mg \cdot g^{-1}$)	References
Brown alga *cystoseira barbatula* Kützing	38.61 $mg \cdot g^{-1}$ at 35°C	Caparkaya and Cavas, 2008
Chaetophora elegans algae	333 $mg \cdot g^{-1}$ at 30°C	El Jamal and Ncibi, 2012
Carolina	55 $mg \cdot g^{-1}$ at 19°C	Hammud et al., 2011
Green alga *Uva latuca*	40.2 $mg \cdot g^{-1}$	El Sikaily et al., 2006
Chlorophyta hydrodictyon africanum	124.11 $mg \cdot g^{-1}$ at 25°C	Current study
Scollymus Hispanics L.	263.92 $mg \cdot g^{-1}$	Barka et al., 2011

algae (El-Batal et al., 2012). Different algae species have been tested of their capability to adsorb dyes (Daneshvar et al., 2012; Akar et al., 2009; Igbal and Saeed, 2007; Padmesh et al., 2005). Moreno-Garrido (2008), described current micro algae immobilization techniques and applications. The techniques, according to Moreno-Garido (2008) include passive immobilization, chemical attachment, active immobilization, silica gel entrapment, use of synthetic polymers etc. Entrapment of biosorbents improves mechanical properties and reduces problems associated with clogging (de-Bashan and Bashan, 2010; Kanchana et al., 2014).

In this study, we report a potentially viable approach for the removal of methylene blue (MB) from aqueous solutions using silica gel immobilized *Chlorophyta hydrodictyon africunum*, an algae species that widely thrives in the Zimbabwean summer weather. The chemical structure of methylene blue is shown in Figure 1. Studies carried out by other researchers have demonstrated that alga based adsorbents can be used for the removal of methylene blue with varying success. Table 1 lists some of the examples of methylene blue removal using various alga species.

MATERIALS AND METHODS

Instruments

A Genesys 10S UV/Vis spectrophotometer was used to determine concentrations of dye solutions. An orbital shaker was used to shake adsorbents suspended in dye solutions. pH measurements were taken using a pH meter and Hanna Instruments.

Reagents

Chemicals used in this research were of reagent grade unless otherwise specified. The dye MB was purchased from Saarchem (Pvt) Ltd, (South Africa). Sodium hydroxide and hydrochloric acid were purchased from Skylabs (Pvt) Ltd. (South Africa). Sodium silicate was of technical grade purchased from a local supplier, Zimbabwe Phosphate Industries. All experiments were conducted in distilled water.

Sample preparation

C. hydrodictyon africanum, an algae species that blooms in summer weather, was harvested from Mwenje Dam in Mashonaland Central Province (Zimbabwe), washed with distilled water before being dried at room temperature over a period of 30 days. The dried algae was ground and sieved through a 53-µm sieve. The fine particulate powder was used for immobilization experiments.

Preparation of adsorbent

The immobilization of algae into silica gel matrix was carried out using a method previously reported (Rangasayatorn et al., 2004). 200 to 1000 mg of dry algae biomass was mixed with 25 mL of 6% sodium silicate solution (v/v) and 25 mL of distilled water. With continual stirring, the pH of the solution was reduced to 7.3 by gradual addition of 18% HCl solution (v/v) by which gelling will have started embedding the algae in the process. The gel was aged for 3 days at 40°C. The gel was washed with distilled water and dried at 80°C overnight. The gel was cut into smaller pieces and sieved to remove smaller than 150 µm ones. The immobilization of the algae ranged from 100 to 400 $mg \cdot g^{-1}$ depending on the algae added to a fixed volume of sodium silicate solution. A flow diagram for the general preparation of the immobilized bio sorbent is shown in Figure 2 and a picture for the immobilization step in Figure 3.

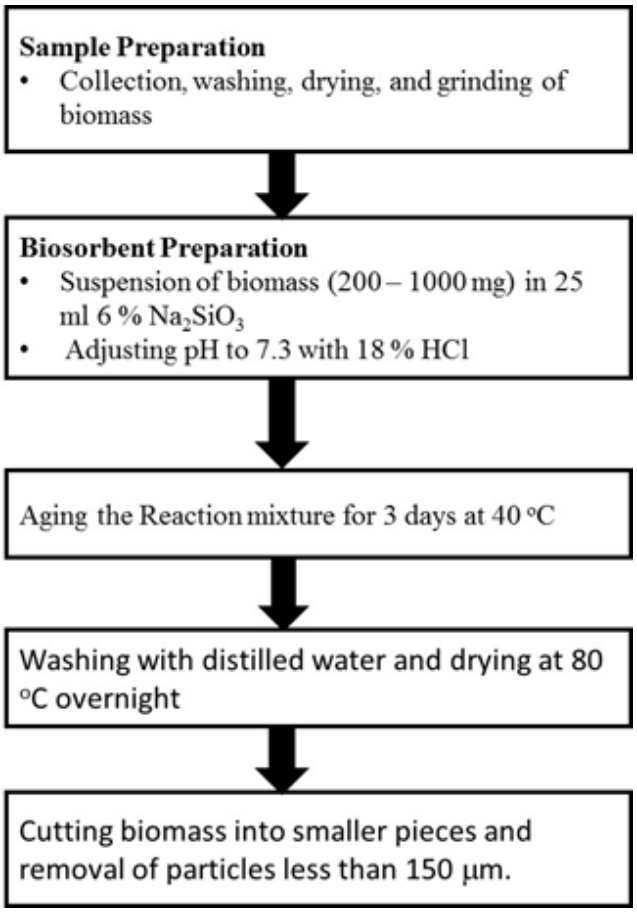

Figure 2. Flow diagram for the preparation of immobilized biosorbent.

Figure 3. Picture of immobilization of *Chlorophyta hydrodictyon africanum* in silica gel matrix.

Characterization of the adsorbent

Adsorption experiments were carried out to determine the effect of pH, algae loading capacity and contact time on the adsorption properties of silica gel immobilized *C. hydrodictyon africanum* adsorbent. This was achieved by mixing 50 mL of 25, 50, 100 and 200 mg·L^{-1} dye solutions with known mass of silica gel embedded algae. The mixture was equilibrated by shaking with an orbital shaker. The initial and final dye concentrations were measured on a Genesys 10S UV/Vis spectrophotometer.

RESULTS AND DISCUSSION

Effect of pH

The effect of pH for the pH range 2-8 at an initial dye concentration of 200 mg·L^{-1} and an adsorbent dosage of 0.8 g·L^{-1} was investigated. At pH>8 the silica begins to dissolve releasing algae. The adsorption capacity of silica gel-immobilized alga was determined from the concentration difference of the solution, at the beginning and at equilibrium using equation (1).

$$q_e = \frac{V(c_i - c_e)}{100 \times m_{ads}} \quad (1)$$

Where, c_i and c_e are initial and equilibrium dye concentration, V the volume of the solution and m_{ads} mass of adsorbent. The results are illustrated in Figure 4. From the diagram, it can be observed that there was a general increase in adsorption capacity with increase in pH for MB. It can be assumed that at lower pH, the surface of algae is positively charged prompting repulsion between the surface and dye molecules. This trend has also been observed by Fernandes et al. (2012) and Rubin et al. (2005) and was attributed to competition between the H^+ and dye cations at lower pH for sorption sites on the immobilized adsorbent resulting in low sorption capacity.

Effect of dosage levels

The effect of adsorbent dosage levels was investigated for the dosage range 100 to 400 mg biomass per g silica. The results illustrated in Figure 5, shows an increase in adsorption up to a dosage of 300 mg biomass per gram silica. No significant increase was observed for dosage levels above 300 mg biomass per g silica. A maximum adsorption of 124.11 mg·g^{-1} was obtained for MB. A high adsorption capacity for basic dyes was also observed by Khataee et al. (2013) on the biosorption of acid orange 7, basic red 46 and basic blue 3.

Effect of initial dye concentration

The effect of initial dye concentration at maximum biosorbent loading capacity was investigated within the 25 to 200 mg·L^{-1} dye concentration range. Figure 6 shows a general increase in adsorption capacity with

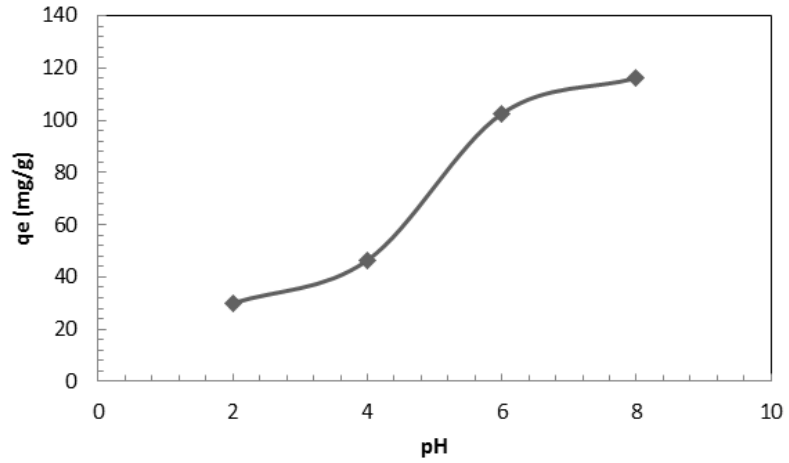

Figure 4. Effect of pH on the adsorption capacity of MB onto *Chlorophyta hydrodictyon africanum* (c_0=200 mg·L^{-1}, t=90 min, agitation speed= 135 rpm).

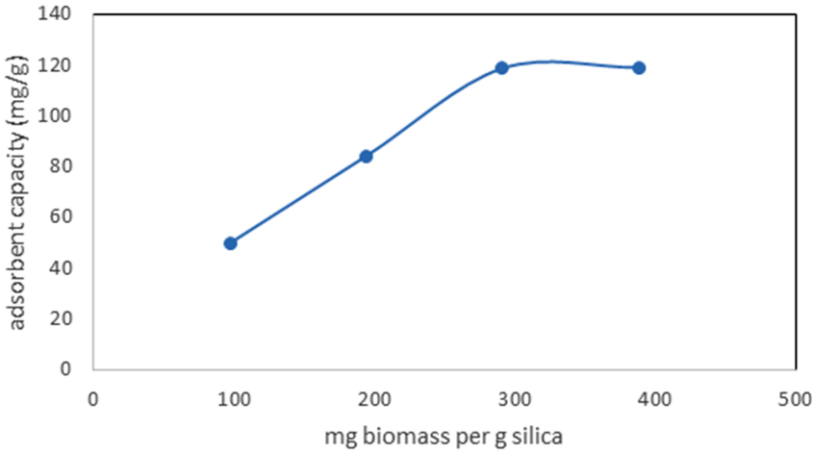

Figure 5. Effect of dosage level on maximum adsorption capacity of MB onto *Chlorophyta hydrodictyon africanum* encapsulated on to silica gel (pH8, t=90 min, T=25°C, agitation speed = 135 rpm).

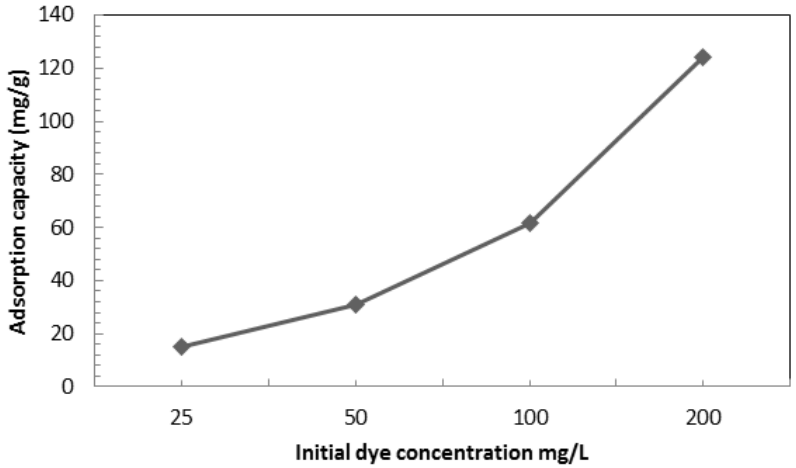

Figure 6. Effect of initial concentration on the adsorption capacity of MB at an adsorbent dosage of 0.8 g·L^{-1} and pH8.

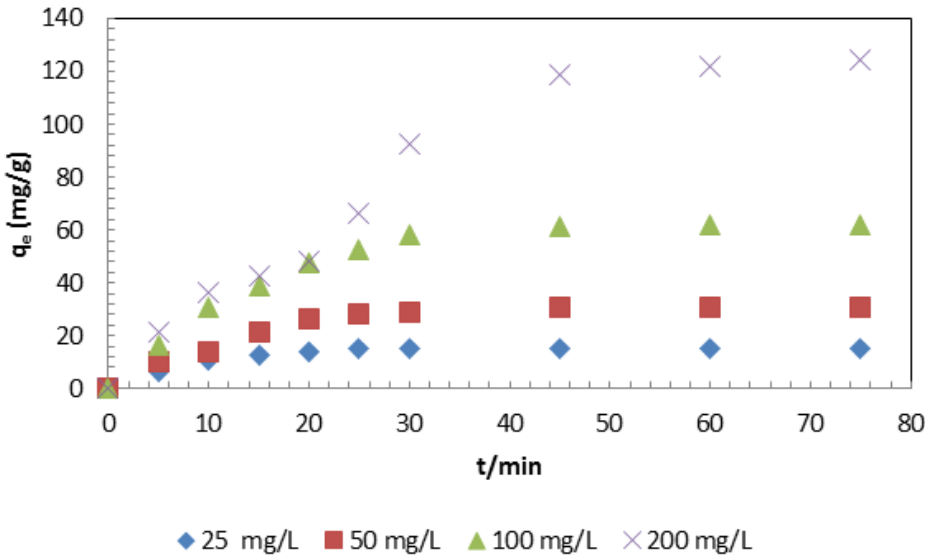

Figure 7. Effect of contact time on adsorption capacity of MB onto silica gel immobilized *Chlorophyta hydrodictyon africanum* for different initial dye concentrations (pH8, T=25°C, agitation speed =135 rpm, adsorbent dosage = 0.8 g·L^{-1}).

increase in initial dye concentration up to a maximum adsorption of 124.11 mg·g^{-1}. Adsorption of MB using *Spirodela polyrrhiza* (Waranusantigul et al., 2003) and *Ulothrix sp*. (Doğar et al., 2010) yielded comparable results.

Effect of Contact time

Contact time determines the adsorbent's sorption capacity. Shorter equilibration times are most desirable for the application of adsorbents. The removal efficiency as function of time and initial dye concentration was investigated for equilibration times of up to 80 min. The result is graphically presented in Figure 7. From the graph, it can be observed that equilibrium was quickly reached in 30 min for initial concentrations of less than maximum adsorption capacity. At higher initial concentrations, equilibrium was reached after 45 min. The results are similar to adsorption experiments with acid dye, acid orange 7 and two basic dyes; Basic Red 46 and Basic Blue 3 carried out by Khataee et al. (2013). Doğar et al. (2010) who carried out similar experiments with MB using the green algae *Ulothrix sp.* biosorbent attributed their observations to concentration gradient between bulk and sorbent surface.

Kinetic studies

Pseudo-first and second order kinetics

Kinetic data were fitted into three models namely the pseudo-first order, pseudo-second and the intra-particle diffusion. Adsorption experiments were carried out using 0.8 g adsorbent which was added to 50 mL of MB. Equilibrium was attained by shaking with an orbital shaker for 90 min at 25°C and an agitation speed of 135 rpm.

The pseudo-first order model is expressed as:

$$\frac{dq}{dt} = k_1(q_e - q) \quad (2)$$

Where, k_1 is the pseudo-first order constant, q_e and q are the amounts adsorbed at equilibrium and after a certain time t, respectively. Assuming that when t=0 and q=0 and integrating the Equation (2) gives:

$$\log(q_e - q) = \log q_e - \frac{k_1}{2.303} t \quad (3)$$

The value of k_1 can be obtained from the slope of a plot of *log* (q_e-q) against t. The results are illustrated in Figure 8.

The pseudo-second order rate equation is expressed as

$$\frac{dq}{dt} = k_2(q_e - q)^2 \quad (4)$$

Where, k_2 is the rate constant of the second-order sorption. The linearized integrated form of Equation 4

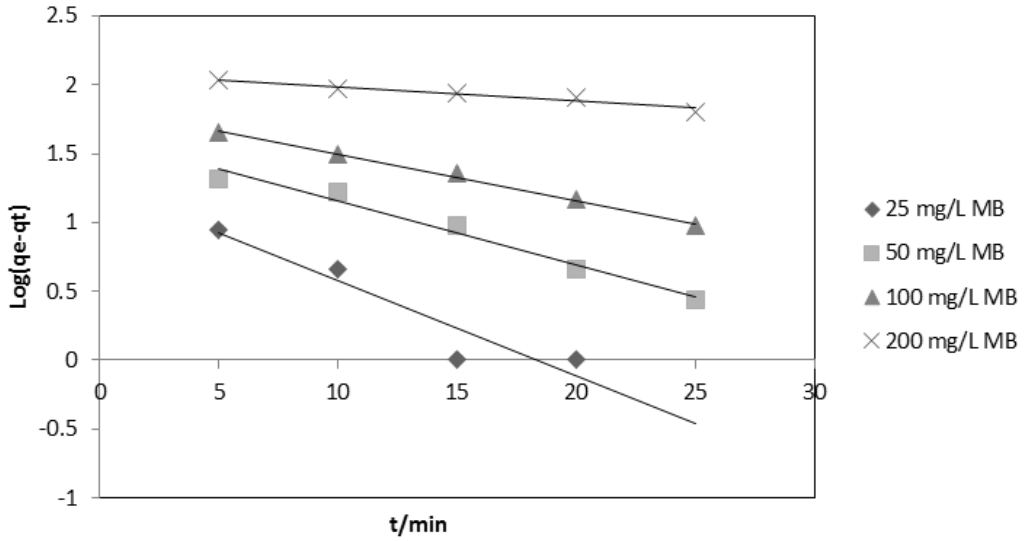

Figure 8. Pseudo first order kinetic modelling for the adsorption of MB on to silica gel immobilized *Chlorophyta hydrodictyon africanum* (pH=8, agitation speed = 135 rpm, T=25°C).

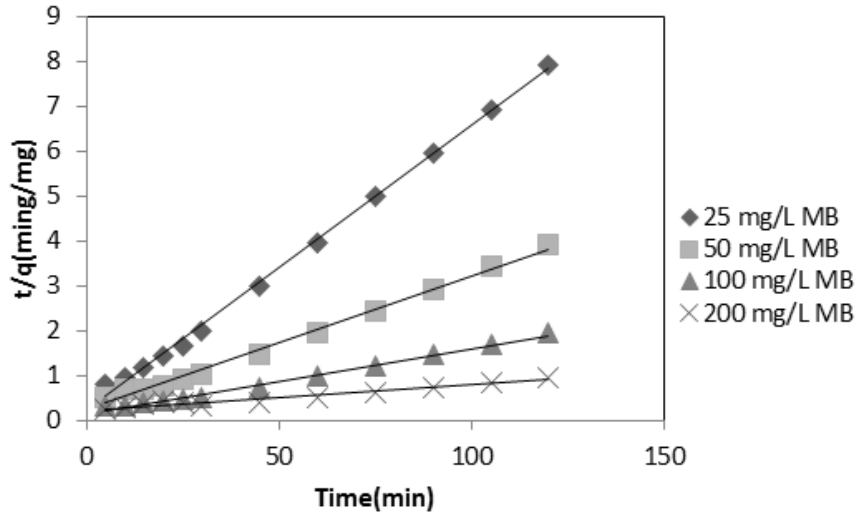

Figure 9. Pseudo second order kinetic modelling of adsorption of MB onto silica gel immobilized C*hlorophyta hydrodictyon africanum (*pH=8, agitation speed = 135 rpm, T=25°C).

is given as

$$\frac{t}{q} = \frac{1}{k_2 q_e^{\,2}} + \frac{1}{q_e} t \qquad (5)$$

The rate parameters k_2 and q_e can be obtained directly from the intercept and slope of the plot of *t/q* versus t. The results for the plot are illustrated in Figure 9. Parameters for both pseudo-first order and pseudo second order kinetics are shown in Table 2. The results show that adsorption processes for MB are better described by a pseudo-second order kinetics with R^2>0.99 in most cases.

Intra-particle diffusion

The intra-particle diffusion or the Weber and Morris model describes processes involved in the sorption of the sorbate by sorbent (Wang et al., 2008; Qui et al., 2009). These include transport of the solute molecules from the aqueous phase to surface of sorbent particles and diffusion of the solute molecules into the pores of

Table 2. Pseudo-first and second order parameters for adsorption of MB on silica immobilized *Chlorophyta hydrodictyon africanum.*

Concentration ($mg \cdot L^{-1}$)	Pseudo first order parameters			Pseudo second order parameters		
	K_1	q_e	R^2	K_1	q_e	R^2
25	0.1601	18.797	0.8913	4.7824	15.6986	0.9979
50	0.1069	41.5528	0.9772	4.3309	33.4448	0.9938
100	0.0783	69.0558	0.9957	7.1327	68.4932	0.9933
200	0.0241	123.2537	0.9363	4.761	172.4138	0.9430

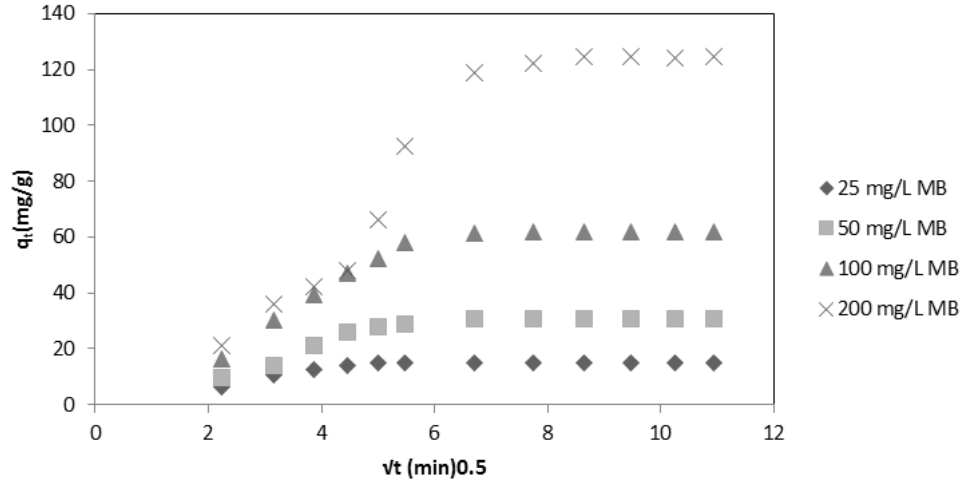

Figure 10. Intra-particle diffusion plot for the adsorption of MB using immobilized *Chlorophyta hydrodictyon africanum* (pH 8, adsorbent dosage = 1.6 $g \cdot L^{-1}$ at 25°C).

Table 3. Intra-particle diffusion parameters for the adsorption of MB on *Chlorophyta hydrodictyon africanum* immobilized of silica gel.

Dye concentration ($mg \cdot L^{-1}$)	k_{id1}	R^2 value	k_{id2}	R^2 value
25	3.1602	0.9718	0.198	0.3685
50	7.0371	0.9788	0.3872	0.6043
100	13.044	0.9961	0.5174	0.5100
200	14.728	0.9423	1.1918	0.7030

sorbent. The later step is a very slow process. Intra-particle diffusion processes can be described by equation (6).

$$q = k_{id}\sqrt{t} + c_{id} \quad (6)$$

Where, k_{id} ($mg \cdot g^{-1}min^{-0.5}$) is the intra-particle diffusion rate constant and c_{id} is the intercept. In the intra-particle diffusion model, the intercept helps to predict the effect of the boundary layer on the sorption process. The larger the intercept, the greater the boundary layer effect. These parameters can determined from a plot of q against $\sqrt{t}$. A plot of intra-particle diffusion is illustrated in Figure 10. The diagram shows two distinct zones. The first portion of the plot can be attributed to bulk diffusion and the other portion to intra-particle diffusion. The k_{id1} and k_{id2} values are shown in Table 3. The k_{id1} values are as expected greater than k_{id2} values.

Equilibrium studies

Adsorption isotherms were used for the design of adsorption systems and study of surface properties of sorbents. Isotherms that were used are the Langmuir and

Table 4. Isotherm parameters for the decolorization of MB using *Chlorophyta hydrodictyon africanum* fixed on to silica gel.

Dye	Langmuir constant			Freundlich		
	$mg \cdot g^{-1}$	K_L ($L \cdot mg^{-1}$)	R^2	$K_F(mg^{1-1/n}L^{1/n}g^{-1})$	*n*	R^2
MB	227.27	0.2990	0.9898	0.5810	1.9870	1.00

R^2 values show that MB adsorption can accurately be described by the Freundlich model than the Langmuir model although both models could be used as evidenced by R^2 values of 1.00 and 0.9898, respectively.

Freundlich. The Langmuir assumes a monolayer homogeneous sorption site while the Freundlich assumes a heterogeneous sight. Table 4 shows isotherm parameters for the removal of MB from synthetic wastewaters. It can be seen that the data fits well to the Freundlich isotherm with R^2 approaching a unit value for MB. The R^2 values for Langmuir isotherms of MB 0.9898. The Freundlich constants K_F and *n* indicate the affinity of the adsorbent towards the biomass. When *n* is greater than 1, there is positive binding and a heterogeneous nature of adsorption. The *n* values of 1.987 indicated a favorable biosorption of MB.

Conclusion

The present study investigated the adsorption of MB from aqueous solutions using silica gel immobilized *C. hydrodictyon africanum.* This adsorbent has been demonstrated to be a highly effective material for the adsorption of MB from aqueous solutions. The adsorption capacity was found to be strongly pH dependent; adsorption capacity increased from about 30 $mg.g^{-1}$(pH=2) to about 124 $mg.g^{-1}$ (pH=8) at the biosorbent immobilization of 300 g biomass per gram silica. Adsorption kinetics studies revealed that the adsorption process followed the Pseudo-second order kinetic model. The equilibrium data were described by the Freundlich and the Langmuir isotherm models, but the Freundlich fit the experimental data well, with an R^2 value of 1.00. Value of *n* was greater than 1 confirming that the prepared adsorbent is favorable for adsorption of MB dye. This research showed that the adsorbent, silica gel immobilized *C. hydrodictyon africanum,* could be applied as a possible adsorbent for the removal of MB dye from wastewaters.

Conflict of interests

The authors did not declare any conflict of interest.

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Characterization of immobilized post-carbohydrate meal salivary α-amylase

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Saliva containing amylase was collected from an individual 5 min after a carbohydrate meal and filtered using a dialysis bag to remove starch particles. The filtered saliva was immobilized on calcium alginate beads. The effect of experimental parameters like pH, temperature and substrate concentration on the activity of the immobilized post-carbohydrate meal salivary α-amylase was determined. The immobilized salivary α-amylase had an optimum activity at temperature 40°C and pH 7.0. The activation energy (Ea) as obtained from the Arrhenius plot was 31.4 kJ/Mol. The kinetic parameters Km and Vmax of the immobilized α-amylase were found to be 1.6 mg/ml and 16.4 µmol/min, respectively; this was compared to that of free salivary α-amylase (Km = 0.0048 mg/ml) and α-amylases from fungi and bacteria sources. Immobilization tends to increase the Km of the immobilized enzyme, indicating a low affinity for substrate, however the enzymes Km was lower than that of some microbial α-amylases. The results obtained from the characterization of immobilized post-carbohydrate meal salivary α-amylase in this study show that immobilization had no significant effect on the enzyme and compared to kinetic parameters of microbial α-amylase, immobilized salivary α-amylase may not be of significant benefit as alternative source of α-amylase in the industrial bioprocesses.

Key words: Enzyme activity, carbohydrate, immobilized enzyme, industrial bioprocess, kinetics, salivary α-amylase.

INTRODUCTION

Alpha amylases have been on increasing demand for various industries, due to their applications in the production of wide array of products, ranging from conversion of starch to sugar syrups, textile, paper, brewing, baking, distilling industries, preparation of digestive aids, production of cakes, and the production of cyclodextrins for the pharmaceutical industry (Sivaramakrishan et al., 2006; Gupta et al., 2003; Reddy et al., 2003). These enzymes account for about 30% of the world's enzyme production (Chi et al., 2009; Van der Maarel et al., 2002). Hence, there is enormous interest in developing alpha amylases with better properties. Alpha amylases (endo-1, 4-α-D-glucanglucanohydrolase EC 3.2.1.1) are extracellular endo enzymes that randomly

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Abbreviations: Ea, Activation energy.

cleave α-1,4 linkages between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose and maltotriose units (Uma and Nasrin, 2013). Most of the α- amylases are metallo-enzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability. The α-amylase family can roughly be divided into two groups: the starch hydrolyzing enzymes and the starch modifying, or transglycosylating enzymes (Sobukola and Aboderin, 2012). The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to a number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps (Satyanarayana et al., 2005).

The human buccal cavity (mouth) can be likened to a template of industrial reactor whose salivary amylase may possess some industrial applications when characterized. Although, the quantity of salivary amylase may be small in the mouth, it may possess better kinetic parameters which could be genetically engineered to complement microbial sources of the amylase. The human salivary α-amylases initiate the chemical process of digestion in the mouth and like amylases from other sources; break down large insoluble starch molecules into soluble starches producing successively smaller starch molecules and ultimately maltose. They act only on linear α-1,4-glycosidic bonds, and hence depend on other enzymes like β-amylase and glucosidase for complete hydrolysis of branched products (Sivaramakrishnan et al., 2006; Robert et al., 2006; Uma and Nasrin, 2013).

Research has shown that the enzyme is optimally active at temperature of 37°C and pH range of 6.7 to 7.0 (Vijayaraghavan et al., 2011). However, the enzyme is inactivated in the stomach by gastric juice at pH 3.3 (Agamemnon and Stefan, 2003). The enzyme is a metalloenzyme and thus requires the presence of metallic ions like calcium and sodium for effective catalytic activity (Siddhartha et al., 2013). The aim of this study was to examine the kinetic property of the immobilized human salivary α-amylase as a possible alternative source of industrial enzyme.

MATERIALS AND METHODS

Collection of salivary α-amylase

Saliva (5 ml) was collected from ten (10) individuals 5 min after a carbohydrate meal. The saliva samples were then filtered using a porous material to remove starch particles. About 1.5 g of sodium alginate was dissolved in 50 ml of distilled water and autoclaved at 121°C for 15 min after cooling to room temperature. 4.5 ml of the crude enzyme sample was added and mixed. The mixture was then allowed to stand for 10 min. The enzyme-alginate mixture was carefully pumped through sterile syringes drop wise into beaker containing 250 ml of sterile 0.12 g calcium chloride in order for the mixture to form beads. The beads were kept in solution for 1 h at 4°C to ensure complete precipitation according to a modified method of James (1992).

Determination of the number of beads of immobilized salivary α-amylase with highest activity

The number of beads that gave the maximum activity with time was determined. Six (6) test tubes were used. The first test tube contained 1 bead + 5 ml of 1% starch + 1 ml of NaCl. The second test tube contained 2 beads + 5 ml of 1% starch + 1 ml of NaCl until the sixth test tube with 6 beads. It was found that 2 beads of size 2 mm yielded the maximum activity and hence was used to characterize the immobilized enzyme. Activity of the immobilized salivary α-amylase was defined as the amount of glucose produced in mM/min at a given temperature when the substrate is hydrolyzed by the enzyme.

Effect of substrate concentration, temperature and pH on α-amylase activity

Starch concentration for optimum extracellular amylase activity varied from 5.0 to 30 mg/ml in 0.1 M potassium phosphate buffer (pH 7.5). Kinetic data were transformed into Lineweaver–Burk plots with graph pad prismprogram (version 5.0). The Km value was calculated by slope of the curve. Extracellular α-amylase activity was also performed at different temperatures ranging from 20 to 70°C. Effect of pH was assayed using 0.1 M pH buffer solutions ranging from pH 4.5 to 9 in increments of one pH unit, (Note that phosphate buffer is only good for pH = 4.5 to 9 due to the dissociation constant). All enzyme assay measure either the consumption of substrate or production of products over time. Initial rate experiments were used to measure the amylase activity. When an enzyme is mixed with a large excess of substrate, the enzyme-substrate intermediates build up in a fast initial transient. Then the reaction achieves a steady-state kinetics in which enzyme substrate intermediates remains approximately constant over time and the reaction rate changes relatively slow. Rates are measured for a short period after the attainment of a quasi-steady state, typically by monitoring the accumulation of products with time. Because the measurements are carried for a short period of time also large excess substrate, the assumption that the amount of free substrate is approximately equal to the amount of initial substrate is usually made. The enzyme activity is the measure of the quantity of active enzyme present. There are two ways to measure enzyme activity; monitoring the disappearance of substrate or the appearance of product. Measuring the appearance of the product is usually accurate because detecting small changes in [P] ([P]=0) is easier to measure than detecting small changes in [S].

Amylase activity = moles of substrate per unit time = rate x reaction volume.

RESULTS

The result from the plot of immobilized post carbohydrate meal salivary α-amylase activity against temperatures values ranging from 25 to 70°C showed that temperature effect on the immobilized salivary α-amylase was optima at 40°C (Figure 1). In Table 1, optimal temperatures and activities of immobilized human salivary α-amylase samples were compared with known microbial α-amylases. The result showed that the salivary amylase had significantly ($p<0.05$) lowered optimum temperature than the microbial sources. The pH effect on the immobilized enzyme was also determined within pH values range of 4.5 to 9.0; the enzyme was found to be

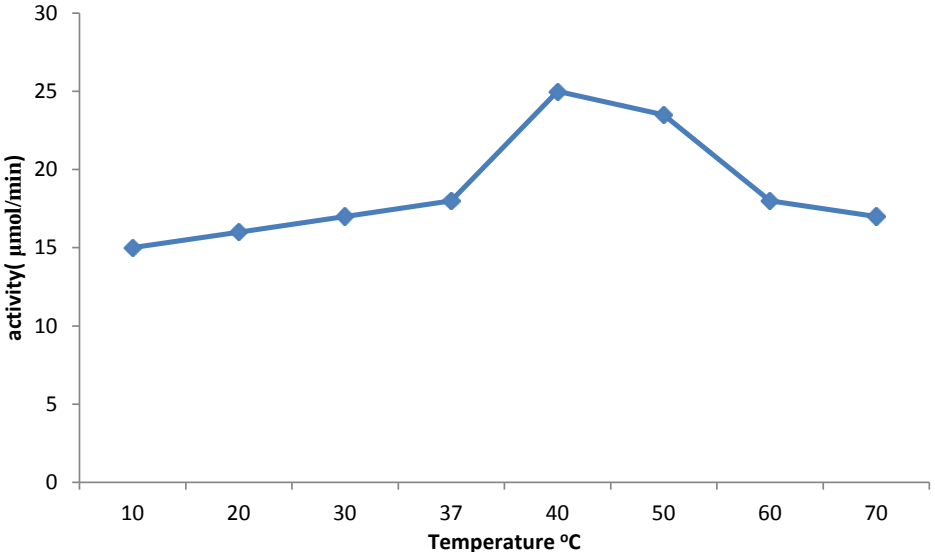

Figure 1. Effect of temperature on human immobilized salivary α-amylase activity showing optimum temperature of 40°C.

Table 1. Comparison between residual activity of human salivary α-amylase and microbial α-amylases at different temperatures.

Organism	Temperature range (°C)	Residual activity (%)	Temperature Optimum (°C)	References
Human salivary (immobilized)	20 - 70	25 (20 min)	40	This study
Bacillus sp. PS1-3	65 - 100	50 (80°C for 2.5 h)	70	Goyal et al. (2005)
Pyrococcus furiosus	80 - 100	50 (98°C for 13 h)	100	Vieille and Zeikus (2001)
Aspergillus tamarii	50 - 60	90 (65°C for 3 h)	55	Moreira et al. (2004)
Lactobacillus manihotivorans	50 - 70	70(80°C for 2.5 h)	55	Aguilar et al. (2000)

optimally active at pH 7.0 (Figure 2); this was compared with optimal pH values of fungal and bacteria α-amylases as shown in Table 2. The activation energy of the immobilized enzyme obtained from an Arrhenius plot was 1.4 kJ/mol (Figure 3). The Km and Vmax of the enzyme were 1.6 mg/ml and 16.4 µmol/min, respectively, as obtained from a Line weaver-Bulk plot (Figure 4).

DISCUSSION

In spite of the wide distribution of α-amylases from fungal and bacterial sources, the diversity of its industrial application creates the need to search for novel α-amylases with novel and improved properties. The rate of hydrolysis of starch by α-amylase depends on many process conditions such as temperature, pH, nature of substrate, substrate concentration, enzyme concentration, presence of Ca^{2+} and other stabilizing agents. α -amylases with properties suitable for industrial conditions and applications have to be appropriately selected based on their high demand. Results obtained from this research work showed that the immobilized salivary α-amylase activity increased as temperature was increased from 20°C. This initial rise in temperature could be due to the increase in probability of effective collision between the reactants as a result of an increase in their average kinetic energy (Okoye et al., 2013). The optimal temperature was obtained at 40°C (Figure 1), followed by a sudden decrease in the activity with further increase in temperature. It is desirable that α-amylases be active at high temperatures of gelatinization (100 to 110°C) and liquefaction (80 to 90°C) to economize the industrial bioprocess; α-amylases from microbial sources seems to be more thermostable (Table 1) than the immobilized salivary α-amylase as observed in this research work (Haki and Rakshit, 2003).

Similarly, the results also showed that the activity of the immobilized enzyme was affected by changes in pH. As the pH of the medium was increased, the activity of the enzyme also increased up to an optimum value of 7.0 (Figure 2). Further increase in pH resulted to a fall in activity and this could possibly be due to ionization of amino-acid side chains that are involved in the catalytic

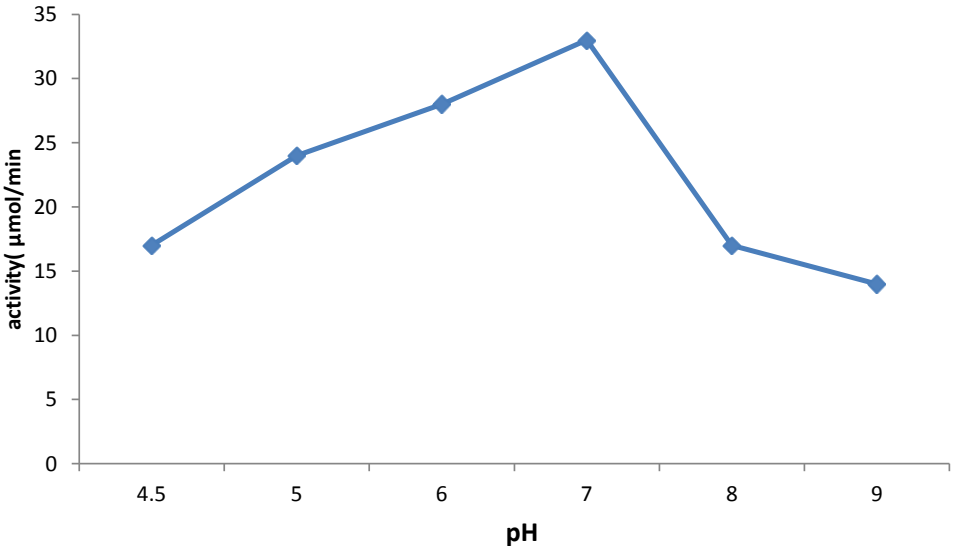

Figure 2. Effect of pH on the activity of human immobilized salivary α-amylase showing optimum pH at 7.

Table 2. Comparison between residual activity of human salivary α-amylase and microbial α-amylases at different pH.

Organism	pH range	Residual activity (%)	pH optimum	Reference
Human saliva (immobilized)	4.5 - 9	32 (20 min)	7.0	This study
Bacillus sp. PS-7	5.0 - 8	96 (pH = 5.0 for 90 min)	6.5	Sodhi et al. (2005)
Bacillus sp.PS1-3	5.0 - 5.5	55 (pH = 10 for 15 h)	5.5	Goyal et al. (2005)
Bacillus sp. *ANT-6*	9 - 13	90 (pH 2.0 for 30 min)	10.5	Burhan et al. (2004)

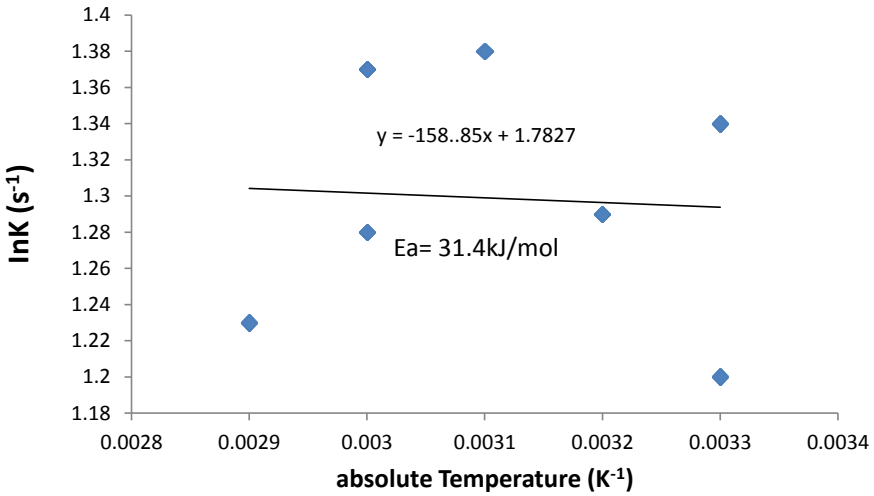

Figure 3. Arrhenius plot of human immobilized salivary α-amylase showing activation energy.

mechanism or may reflect an onset of denaturation of the enzyme (Sundarram and Murthy, 2014). A comparison of the optimum pH of the immobilized salivary a-amylase to those of microbial α-amylases showed that some

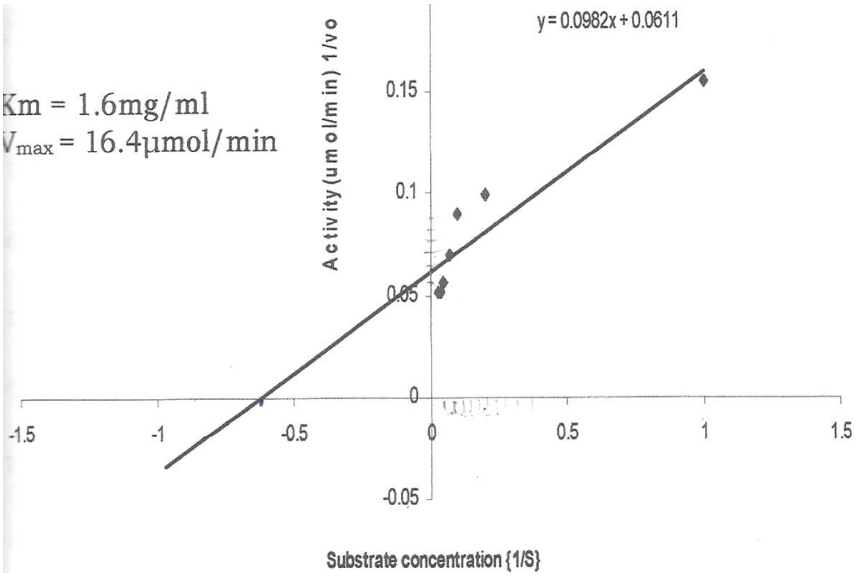

Figure 4. Line Weaver Burk plot of immobilized salivary α-amylase showing Km and Vmax values

Table 3. Comparison of Km values of immobilized salivary α-amylase and α-amylases from microbial sources.

Human/microbial species	Km (mg/ml)	Reference
Human saliva (immobilized)	1.6	This study
Bacillus sp. PB3224	2.7	Orlando et al. (1983)
B. subtilis	3.8	Ciobanu et al. (1976)
B. subtilis 65	1.2	Hayashida et al. (1988)
Bacillus sp. KCC103	1.9	Nagarajan et al. (2006)
truncated *Bacillus subtilis* alpha-amylase in *E. coli*	14	Marco et al. (1996)

microbial amylases are optimally active under acidic conditions, some under basic while others in a neutral medium. The activation energy (Ea), of the reaction was also investigated and found to be 31.4 kJ/ml (Figure 3). This is the minimum energy reactants, must possesses to overcome the activation barrier. The role of the enzyme is to reduce this barrier, thereby increasing the rate of reaction, hence the formation of products. From the Arrhenius plot, Ea is best regarded as the experimentally determine parameter that indicates the sensitivity of a reaction rate to temperature. Enzymes facilitate the rate of catalysis by providing an alternative route with less Ea for reacting species. Hence, Ea (31.4 kJ/mol) in this study is the energy required to hydrolyse starch to glucose molecules. More energy will have been required if the reaction was devoid of an enzyme.

The kinetic parameters Km and Vmax of the immobilized enzyme were obtained from a Lineweaver-Burk plot using starch as the substrate. The Km and Vmax were 1.6 mg/ml and 16.4 µmol/min, respectively. The value of the immobilized enzyme Km was lower than Km values from microbial sources (Table 3). The Km of an enzyme, relative to the concentration of its substrate under normal conditions permits prediction of whether or not the rate of formation of product, will be affected by the availability of the substrate. Since, Km is the major factor determining the enzymes' affinity for its substrate, which also affect the rate at which the enzyme is saturated by it substrate (Das et al., 2011) an enzyme with low Km has high affinity for its substrate and vice versa.

In conclusion, from the results obtained from immobilization of α-amylase from human saliva compared to that from microbial sources, immobilized salivary α-amylase is not thermally stable and may not be active in an acidic or basic medium though it has a low Km, signifying that the enzyme may have high affinity for its substrate starch which is of significant advantage to the industrial processes. Most industrial processes operate at very high temperature and during bioprocesses lot of acid and basic metabolites are released that alters the pH of

the bioreactor which may result to loss of enzyme activity. However, amylases from some microbial sources can operate in both acidic and basic medium and thus immobilized salivary α-amylase may not be a perfect alternative for industrial purposes.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effect of *DGAT1* gene mutation in sows of dam-line on the composition of the produced milk and piglet rearing during 21-day lactation

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Diacylglycerol acyltransferase 1 gene (*DGAT1*) involved in the synthesis and transport of triglycerides is located on chromosome 4 in pigs, in the region with about 200 QTLs responsible among other things for: fat thickness, daily gains, fat content and composition of fatty acids. It is thus probable that the gene polymorphism (as in cows) may affect the fat content in colostrum and milk of sows. The objective of the experiment was to assess the effect of *DGAT1* polymorphism on the milk composition of sows and as a result on piglet rearing during the suckling period. The experiment was performed on 207 sows of breeds used in breeding program as a dam-line: polish large white (PLW) and polish landrace (PL). Colostrum and milk of sows were collected at 1, 7, 14 and 21 days of lactation to assay solids, total protein, fat and lactose. Data on piglet rearing performance were collected at 1, 7, 14 and 21 days of lactation. The tests performed showed that A/G rs45434075 *DGAT1* mutation occurring in PLW and PL sows did not significantly affect the quality of their colostrum and milk expressed as solids content, and at the same time on piglet rearing performance. However, it was observed that PLW sows of the *DGAT1*^{GG} genotype was characterised by a higher fat content in colostrum, whereas PL sows of the same genotype had an increased protein content and a reduced lactose content in milk.

Key words: *DGAT1*, Polymorphism, milk, rearing of piglets, maternal breed.

INTRODUCTION

The effect of gene mutations on the chemical composition of porcine milk has not been widely studied so far. There are few publications on this subject, and they suggest certain correlations between genotype of sows and milk composition. In their studies on transgenic sows, Monaco et al. (2005) revealed that increased mammary gland expression of the *IGF1* gene (lactation regulator) did not cause an increase in the amount of the

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Abbreviations: PLW, Polish large white; **PL,** polish landrace.

produced milk or a change in its composition. Babicz (2008) analysed the effect of polymorphism of selected genes on the level of chemical components of colostrum and he observed that polymorphisms of *RYR1/HinfI, GH/MspI, PRL/HpyCH4III* genes significantly affected protein content of milk in Puławska sows. Szyndler-Nędza et al. (2013) investigated the effect of *MC4R*(G1426A) and *LEPR/HpaII* polymorphisms on the composition of colostrum from PLW and PL sows which demonstrated that analysed polymorphisms had a similar effect in both breeds. The $MC4R^{A}$ and $LEPR^{B}$ alleles reduce the content of fat, protein and solids in the colostrum of sows. Another interesting gene in this respect might be diacylglycerol acyltransferase 1 (*DGAT1*), whose expression in mammals occurs mainly in the small intestine. This gene participates in intestinal synthesis of triglycerides and their transport to the lymphatic system (Nagalski and Bryła, 2007). Studies conducted on cattle revealed that *DGAT1* polymorphism (K232A-replacement of lysine by alanine) had a significant influence on milk yield and composition (Grisart at al., 2002; Thaller et al., 2003; Kuehn et al., 2007; Streit et al., 2011). Cows with lysine variant in position 232 aa sequence of the *DGAT1* protein were characterised with a higher content mainly of milk fat, but also of protein. The effect of this mutation was confirmed in three subsequent lactations of cows (Thaller et al., 2003). Moreover, studies on Charolaise, Limousin and Retina cattle (Aviles et al., 2013) showed that K232A polymorphism of the *DGAT1* gene affected thickness of cattle fat cover.

Previously, literature has not reported publication concerning the QTL associated with the porcine milk composition and piglet rearing during lactation. Nonneman and Rohrer (2002) and Szczerbal et al. (2007) mapped *DGAT1* gene in pigs and demonstrated that it was located on chromosome 4, in the region with about 200 QTLs responsible for: fat thickness, weight gains, fat content, composition of fatty acids, etc. Cui et al. (2011) analysed *DGAT1* and *DGAT2* gene expression in the longest muscle (*longissimus muscle dorsi*), liver and fat of three different pig breeds. Its correlation with backfat thickness, showed that the highest expression of *DGAT1* gene occurred in the liver and it was positively correlated with this trait ($P \leq 0.05$). Therefore, *DGAT1* polymorphism in pigs (similarly as in cows) may affect the fat content in colostrum and milk of sows.

The objective of the present study was to assess the effect of *DGAT1* polymorphism g. 32748 A>G (NW 003534570.2) identified in intron 2 on the milk composition of sows and its influence on the piglet rearing during the suckling period.

MATERIALS AND METHODS

The studies were performed on 207 sows, including 107 PL sows (58 sows in the second lactation and 49 sows in the third lactation) and 100 PLW sows (53 in the second lactation and 47 in the third lactation). All sows were kept at the Experimental Station of the National Research Institute of Animal Production Ltd. in Żerniki Wielkie, Poland. Sows used for reproduction were maintained under the same feeding and housing conditions. The feeding system is compliant with the farm standards and it was adopted according to various reproductive stages of sows (sows in early gestation, in late gestation, in lactation). In the experiment, only sows in similar condition on the day of mating were used. Their condition was determined based on body weight and last rib (P2) backfat thickness, measured with an ultrasonic device (Piglog 105). Blood was collected from all sows to determine the polymorphisms of *DGAT1* gene. Samples of colostrum and milk were collected from sows during the second and third lactation. After 1 h following delivery the colostrum was milked and collected, and in turn milk 2 h following the morning feeding on 7, 14 and 21 day of lactation. Samples of colostrum and milk were collected from the first, third and sixth teat in the total amount of 50 ml (one sample of milk). The samples were then labelled and cooled to 4°C. Cooled samples of fresh milk were supplied to the Laboratory of Milk Assessment and Analysis of the Wrocław University of Environmental and Life Sciences in order to determine basal composition of the milk. Solids, total protein, fat and lactose were determined in colostrum and milk.

The determinations were performed with the instrument Milko-Scan 133 B by Foss Electric, with the use of infrared analysis according to the enclosed application by Foss. Reproductive performance of the experimental sows was determined based on 207 L, which were analysed for the number and body weight of piglets at birth and at 7, 14 and 21 days of age.

DNA was isolated from leukocytes with the use of the Genomic Wizard Purification Kit (Promega, Madison, WI, USA). Mutation in *DGAT1* gene was determined with PCR-RFLP methods according to Nonneman and Rohrer (2002), with modification of primer pair, which were designed in Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Forward-GCATCCTGAATTGGTGTGTG and Revers - GGCCATTCAGAACAG primers amplified a 257 bp PCR product, which was then digested with *Ava*II restriction enzyme, which recognizes *A/G* rs45434075 (g. 32748 A>G (NW 003534570.2)) substitution. The data were statistically analysed using the procedures of Statisticaver.10 (2011, StatSoft Inc.). The differences between groups were estimated by ANOVA analysis according to the following statistical model:

$$Y = a_i + b_j + c_k + d_l + e_{ijkl}$$

Where, ai = breed (i = 1,2), bj = lactation (j=1,2), ck= lactation period (k=1-4), dl = genotype (l= 1-3). Differences between the means of individual traits were tested at 0.05 and 0.01 P-value using Duncan's multiple range test.

RESULTS AND DISCUSSION

In comparison with Polish Landrace sows (Table 1), Polish Large White sows had a slightly higher body weight on the day of mating and statistically significantly thinner backfat ($P \leq 0.05$). Differences observed in backfat thickness between PLW and PL breeds are compliant with findings presented by Eckert at al. (2013) for sows of these breeds in the national population. Also, Knecht et al. (2014) performing measurements with an ultrasonographic device Aloka SSD-500, showed that PL sows had significantly higher backfat thickness at P2 ($P \leq$

Table 1. Means and standard deviations of body weight and fat thickness on day of mating in PLW and PL sows.

Parameter	PLW		PL		P- value of
	Mean	SD	Mean	SD	PLW/PL
Mean body weight on day of mating (kg)	192.99	27.62	186.34	23.98	0.051
Mean backfat thickness on day of mating P2 (mm)	13.25	3.53	14.57	3.67	0.005

Table 2. Genotype and allele frequency of *DGAT1* gene.

Breed	n	*DGAT1*			Allele frequency		HWE
		AA	*AG*	*GG*	*A*	*G*	P-value
PLW	100	47.00	45.00	8.00	69.5	30.5	0.54
PL	107	25.23	55.14	19.63	52.80	47.20	0.25

0.05) in comparison with PLW sows. Sows of both breeds were characterised with a the lowest genotype frequency of *DGAT1*^{GG} gene (Table 2). In PLW breed, the most frequent were individuals with *DGAT1*^{AA} genotype (47%). The least frequent were homozygous *DGAT1*^{GG} individuals (8%). In PL breed, the most frequent were heterozygous *DGAT1*^{AG} individuals (55.14%). Similarly to PLW, the least frequent were individuals with *DGAT1*^{GG} genotype (19.63%). Both analyzed populations were in HW-equilibrium, it suggests that polymorphism *A/G* rs45434075 of *DGAT1* gene is not associated with pig traits, which were considered during selection in order to improve their reproductive performance. Differences between breeds in the frequency of alleles for *A/G DGAT1* polymorphism were also reported by Mercadé et al. (2005). They showed that *A* allele was not present in the Iberianpigs as well as in the Landrace population, in contrast to the Meishan breed where *A* allele was observed with 35% frequency.

The analysis of the effect of rs45434075 *DGAT1* gene polymorphism on the backfat thickness and body weight of sows on day of mating prior to second and third litter showed no statistically significant differences among the analysed genotypes of *DGAT1* gene (Tables 3 and 4). Sows of both breeds, representing each of the polymorphic forms of *DGAT1* gene, had similar values for these traits; therefore it was not necessary to include these factors in the statistical model. Different results were obtained for cattle. Studies conducted on Charolaise, Limousin and Retina breeds showed that Lys232 variant of *DGAT1* protein increased fat cover thickness of cows, in contrast to Ala232 variant. The authors also showed that homozygous CC (Lys, Lys, KK) cows, with only alanine variant in *DGAT1* protein were the least common in the analysed populations (Aviles et al., 2013). The basic objective of the present study was to assess the effect of *DGAT1* gene polymorphism on basic composition of colostrum and milk and on possible differences in piglet rearing in maternal breeds used in Polish breeding. Results of the analysis are presented in Table 3 for PLW sows and in Table 4 for PL sows.

It can be concluded from the results obtained, that the A/G rs45434075 DGAT1 gene polymorphism in both breeds had an effect on changes in basic components of colostrum and milk. However, this effect was different in each breed. A statistically significant correlation was observed, between *DGAT1* genotype and fat content in colostrum of PLW sows and between the content of protein and lactose in milk of PL sows. PLW sows with *DGAT1*^{GG} genotype had significantly more fat in colostrum (by 1.86%, P≤0.05) than sows with *DGAT1*^{AA} genotype. The obtained results without significances may have been caused due to the small statistical groups representing *DGAT1*^{GG} (8 sows). PL sows with *DGAT1*^{GG} genotype were reported to have significantly higher protein content (by 1.01%, P≤0.05) and significantly lower lactose content in milk (by 0.63% P≤0.05) than individuals with *DGAT1*^{AA} genotype. Differences in the effect of the analysed polymorphism on milk composition between breeds were also observed in cattle. Suchocki et al. (2010) showed that *DGAT1* polymorphism had a more significant influence on the majority of milk components in Jersey cows than in Holstein-Friesians cows. They also observed epistatic effect between *LEPR* and *DGAT1* gene polymorphisms on milk composition of Holstain-Friesians cows. On the other hand, Marchitelli at al. (2013) showed that in Jersey cows, *DGAT1* polymorphism affected only the fat content in milk.

Piglet rearing performance during lactation is affected by numerous factors for example higher milk yield and higher content of basic milk components (Skrzypczak et al., 2012b; Schmidely et al., 2002; Barłowska et al., 2007; Buczyński et al., 2008; Babicz et al., 2011). Studies on the effect of milk protein polymorphism in sows (Skrzypczak et al., 2012b) on piglet rearing performance showed that sows of *CSN1S1*^{AA} and *CSN2*^{BB} genotypes

Table 3. Means and standard deviations for sows condition on the day of mating, chemical composition of colostrum and milk from 21-day lactation, and rearing performance of piglets from sows of PLW breed with different *DGAT1* genotypes.

Parameter			*DGAT1* PLW		
			AA **47**	***AG*** **45**	***GG*** **8**
Condition	Body weight at mating (kg)		195.00±27.91	192.78±26.31	197.00±38.08
	Backfat thickness (P2) (mm)		13.36±4.04	13.16±3.44	13.38±1.99
Composition of colostrum and milk from 21-day lactation	Fat (%)	Colostrum	3.47a±1.63	3.60±2.97	5.33a±2.09
		Milk	7.08±1.13	6.97±1.06	7.13±1.08
	Protein (%)	Colostrum	14.72±3.65	12.76±2.44	13.42±2.31
		Milk	5.03±1.31	4.99±1.32	4.61±0.09
	Lactose (%)	Colostrum	2.33±0.76	2.75±0.82	2.65±0.40
		Milk	5.49±0.67	5.46±0.57	5.79±0.31
	Solids (%)	Colostrum	21.45±3.21	19.92±2.84	21.70±3.59
		Milk	17.97±1.73	17.84±1.33	17.82±0.87
Rearing of piglets during 21-day lactation	Number of piglets at days of age (head)	1	11.67±1.17	11.93±0.75	11.50±1.07
		7	11.04±1.33	11.07±1.01	10.88±1.13
		14	10.39±1.82	10.67±1.13	10.63±1.30
		21	10.06±1.91	10.31±1.12	10.38±1.19
	Weight of piglets at days of age (kg)	1	1.48±0.17	1.43±0.12	1.55±0.18
		7	2.60±0.32	2.66±0.32	2.75±0.33
		14	3.94±0.45	4.11±0.50	4.16±0.39
		21	5.66±0.65	5.72±0.51	5.88±0.48
	Weight gain of piglet until day (kg)	7	1.12±0.28	1.24±0.31	1.20±0.31
		14	2.45±0.43	2.68±0.48	2.61±0.33
		21	4.17±0.62	4.29±0.47	4.33±0.47

Means in rows with the same small letters differ significantly at P≤0.05.

Table 4. Means and standard deviations for sows condition on the day of mating, chemical composition of colostrum and milk from 21-day lactation, and rearing performance of piglets from sows of PL breed with different *DGAT1*genotypes.

Parameter			*DGAT1* PL		
			AA **27**	***AG*** **59**	***GG*** **21**
Condition	Body weight at mating (kg)		189.15±28.03	185.3±23.32	186±20.97
	Backfat thickness (P2) (mm)		15.5±4.65	14.42±3.23	14.29±2.43
Composition of colostrum and milk from 21-day lactation	Fat (%)	Colostrum	5.50±1.71	5.32±2.46	4.10±1.72
		Milk	7.23±0.68	7.28±1.03	7.09±1.65
	Protein (%)	Colostrum	16.05±3.09	14.74±2.67	14.57±3.18
		Milk	4.51a±0.37	4.70±0.84	5.52a±2.50
	Lactose (%)	Colostrum	1.80±0.81	2.38±2.78	2.13±0.93
		Milk	5.74a±0.26	5.52b±0.55	5.11ab±1.01
	Solids (%)	Colostrum	23.79±2.66	22.58±3.11	21.37±3.13
		Milk	17.81±0.86	17.82±1.39	18.07±1.62

Table 4. Contd.

Rearing of piglets during 21-day lactation	Number of piglets at days of age (head)	1	11.00±1.90	11.51±1.17	11.76±0.54
		7	10.56±1.85	10.93±1.26	11.23±0.83
		14	10.33±1.84	10.61±1.29	10.90±0.83
		21	10.04±1.74	10.29±1.38	10.52±1.66
	Weight of piglets at days of age (kg)	1	1.51±0.11	1.48±0.15	1.49±0.13
		7	2.86±0.37	2.78±0.35	2.75±0.33
		14	4.18±0.48	4.18±0.46	4.26±0.62
		21	5.82±0.65	5.66±0.57	5.83±0.62
	Weight gain of piglet until day (kg)	7	1.35±0.32	1.30±0.34	1.26±0.34
		14	2.67±0.42	2.70±0.46	2.77±0.62
		21	4.31±0.59	4.18±0.57	4.34±0.61

Means in rows with the same small letters differ significantly at $P \leq 0.05$.

of casein genes produced more milk, and as a result achieved higher piglet rearing performance, that is, higher body weight and fast body weight gain. Similar studies conducted in goats (Schmidely et al., 2002; Barłowska et al., 2007) showed that some variants of both casein genes affected higher protein and fat content in milk, which was translated into better rearing performance of offspring. In studies on sows of maternal breeds, Babicz et al. (2011) showed that a higher protein and solids content in milk might be one of the factors affecting increased piglet growth and body weight during 21-day lactation. Studies on sows of Złotnicka White breed (Buczyński et al., 2008) concerning the effect of milk composition on piglet rearing performance showed that daily gains in 21th-day of lactation were higher in piglets of sows with a higher fat content in milk (over 7 %). Another factor influencing piglet body weight gain during lactation is milk pH and somatic cells content (Skrzypczak et al., 2012a). The authors of this paper showed negative correlation coefficients among these parameters in sows' milk and piglet body weight in 21th-day of lactation.

Differences observed in the present studies in individual milk parameters of both breeds were not big enough to use the *DGAT1* polymorphism as a genetic marker in selection of solid milk content in investigated sows of dam-line. Therefore, it may be concluded that *A/G* rs45434075 of *DGAT1* mutation, does not change basal milk composition in sows of both breeds and does not also affect piglet rearing performance. The analysis of changes in piglet body weight and weight gain during 21 days of lactation in relation to *DGAT1* genotype of both breeds (Tables 3 and 4) show that there were no statistically significant differences in the analysed traits between polymorphic forms of this gene. The investigated polymorphism g.32748A>G, NW003534570.2 in the second intron was identified in 2002 by Nonneman and Rohrer and added to GenBank in 2004. Recently, SNPdb was added as new interesting missense mutations located in 1, 5 and 7 exon, which could be tested in further study. It can be observed that the last mutation changing aa in protein domain is associated with membrane-bound O-acylo transferase (Ensemble Browser).

Summarising the findings of the performed tests, it can be observed that mutation *A/G* of *DGAT1* gene participating in intestinal synthesis and transport of triglycerides to the lymphatic system - occurring in PLW and PL sows did not significantly affect the quality of their colostrum and milk expressed as solids content, and at the same time on piglet rearing performance. It was only observed that PLW sows of the *DGAT1*^{GG} genotype were characterised by a higher fat content in colostrum, whereas, PL sows of this genotype had an increased protein content and a reduced lactose content in milk. It must be emphasised that the least common in both breeds were individuals with *DGAT1*^{GG} genotype.

Conflict of interests

The authors did not declare any conflict of interest.

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